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(54) Title: METHODS AND COMPOSITIONS FOR TREATMENT OF SOLID TUMORS IN VIVO

(57) Abstract

The present invention provides compositions and methods for treatment of solid tumors with gene therapy utilizing recombinant viral vectors that express polypeptides which selectively initiate irreversible coagulation of blood in the tumor vasculature, inhibit tumor neovascularization, are capable of activating a non-toxic agent into a toxic agent within the tumor vascular wall causing destruction of the vascular bed and absorb or metabolize nutrients in the blood being supplied to the tumor. The production of these polypeptides by transduced cells in or adjacent to the blood vessels of the tumor result in the death of tumor cells.

Methods and Compositions for Treatment
of Solid Tumors *In Vivo*

Technical Field

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The present application relates generally the field of anti-cancer therapy, and more specifically, to methods of killing selected tumor cells using recombinant viral vectors.

Background of the Invention

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Many different therapies are available for treatment of various types of human tumors. These include surgery, radiotherapy, chemotherapy, radioimmunotherapy, and neoadjuvant therapy. These treatments can be used individually or in combination, depending on whether the tumor is metastasizing or non-metastasizing.

15   Surgery is primarily used for non-metastasizing tumors and is therefore the treatment of choice for a variety of potentially curable cancers. In some circumstances, surgery may be used for palliation of noncurable cases or for reconstruction and rehabilitation. In addition, surgery plays a diagnostic role in determining the pathologic staging of the extent of local and regional invasion prior to resection of the primary

20 neoplasm. However, surgery for certain tumor types can be disadvantageous, as the surgery may be disfiguring, disabling, or ineffective. For in non-operable tumors, primary local therapy with ionizing radiation is often the treatment of choice.

Two types of radiation therapy have been used to treat tumors: brachytherapy and teletherapy. In brachytherapy, the radiation source is placed close to the tumor. This

25 intracavitary approach is used for many gynecologic or oral neoplasms. In teletherapy, supervoltage radiotherapy is usually delivered with a linear accelerator, allowing for more precise beam localization and avoiding the complication of skin radiation toxicity. Various approaches are used to increase the radiation dosage to the tumor area while minimizing toxicity to adjacent normal tissues. Radiation therapy is normally delivered in a

30 fractionated fashion, having radiobiologic superiority by permitting time for recovery of normal host tissues (but not the tumor) from sublethal damage during the period of treatment. Fractionated radiation doses are usually administered for 5 days per week until the desired total dose has been delivered, usually over the course of 4-6 weeks.

Unfortunately, radiation therapy is followed by both acute and latent toxicity.

35 Acute toxicity may include generalized fatigue and malaise, anorexia, nausea and vomiting, local skin changes, diarrhea, and mucosal ulceration of the irradiated area. Radiation of large areas, especially the pelvis and proximal long bones, may result in significant bone

marrow suppression. Long-term toxicity may result in hyperpigmentation of the involved skin, decreased function of the irradiated organ, myelopathy, bone necrosis, and secondary malignancies. These toxicities, which are dose-related, and may be minimized or avoided by careful shielding and fractionation.

5   Currently, more than 50% of all patients with cancer receive radiation therapy during the course of their illness. Radiation therapy is frequently the sole agent used with curative intent for certain tumors. For more extensive cancers, radiation is combined with surgery. Radiation is also used as an adjuvant to chemotherapy for some patients with lymphoma or lung cancer and for several cancers in children. Occasionally, chemotherapy
10 may be used to sensitize tumor cells to the toxic effects of radiation.

          Although its use is very limited, locoregional hyperthermia may be used for some tumor sites. Hyperthermia is a form of nonionizing irradiation between 40_ to 42_C that is of value as an adjunct to ionizing irradiation. The treatment may also involve drugs that increase a tumor's response to heat. Hyperthermias greatest use is controlling bulky
15 hypervascular tumors.

          Chemotherapy is used when surgery or radiation is not practical or only partially effective. Chemotherapy, involving the use of cytokines, cytotoxic drugs, hormones, antihormones, and other biological agents, has become an increasingly effective means of treating cancer. The use of a cytokine alone or in combination with other cytokines or
20 cytotoxic drugs has been effective in inducing the regression of metastatic cancers by modulating the immune system (Heaton, K., et al., *Cancer Immunol Immother* 37:213-219, 1993). Useful cytokines include interleukin (IL)-2, IL-4, IL-6, tumor necrosis factor (TNF), α-interferon, and γ-interferon (α-IFN and γ-IFN). Intravenous administration of IL-2 alone has been effective in approximately 15-30% of patients with metastatic renal-cell
25 carcinoma or melanoma and may be efficacious in the treatment of other tumors. Additionally, α-IFN has been shown to be effective against certain solid tumors following resection of the bulk of the tumor tissue, suggesting a possible post-operative role as an adjuvant therapy (reviewed in Heaton, K., et al. *Supra*).

          The combined use of cytokines to increase antitumor efficacy is based on the
30 premise that combining agents that attack neoplastic cells by different mechanisms should increase antitumor effects *in vivo*. Therefore, toxicity should be lower in patients treated with combinations of agents that have different side-effects. Pre-clinical studies have demonstrated that such cytokine combinations can be effective in animal models (reviewed in Heaton, K., et al., *Supra*). Additionally, cytokines can be combined with other cytotoxic
35 agents in order to offer a more effective therapy for neoplastic diseases. Such combinations take advantage of different mechanisms of action and different toxicity profiles. For example, α-IFN appears to potentiate the effects of 5-fluorouracil (5-FU) by increasing

serum levels of active 5-FU metabolites. Also, IL-2 and α-IFN in combination with cisplatin and dacarbazine have produced response rates much higher than any observed with either cytokines or cytotoxic drugs alone.

While most anticancer drugs are used systemically, there are selected indications for
5 local or regional administration. Regional administration involves direct infusion of active chemotherapeutic agents into the tumor site (e.g., intravesical therapy, intraperitoneal therapy, hepatic artery infusion with or without embolization of the main blood supply of the tumor). These treatments can result in significant palliation and improved survival.

However, many patients treated with chemotherapy have a limited response. Such
10 treatment may become ineffective because of drug resistance attributed to spontaneous genetic mutations in subpopulations of cancer cells prior to chemotherapy exposure. Following elimination of the sensitive cells by chemotherapy, the resistant subpopulation grows to become the predominant cell type.

Radioimmunotherapy is a form of treatment that offers the potential for anti-cancer
15 activity with common tumors. Although this therapy is largely experimental, progress is being made in developing useful clinical agents, especially in the hematopoietic malignancies. The success of radioimmunotherapy depends on delivering a large dose of radiation to a selective tumor without injury to radiation sensitive tissue. In the case of solid tumors, a number of radiolabeled polyclonal and monoclonal antibodies have been
20 used in the treatment of patients with hepatoma, intrahepatic cholangiocarcinoma, melanoma, ovarian carcinoma, peritoneal carcinomatosis, and glioma (reviewed in Larson, S., et al., Nucl Med Biol. 21:785-792, 1994). In these cases, [131]Iodine has been the label most commonly used.

The administration of the radiolabeled antibody into a closed space seems to offer
25 the greatest potential for successful treatment. Intravenous administration of the compound is not as effective. A number of monoclonal antibodies have been used, including: M195 (anti-CD-33), a murine monoclonal antibody against hematopoietic progenitors; CC49 (anti-TAG-72), against colorectal, pancreas, ovarian, and other human tumors; and 3F8 (anti-GDZ), localized to primary and metastatic neuroblastomas in
30 patients. However, a substantial problem with this therapy is the development of immunity against murine monoclonal antibodies and associated hematopoietic toxicity.

Neoadjuvant therapy, systemic treatment given before local therapy, has become increasingly more prominent in the treatment of cancer (reviewed in Trimble, E., et al., Cancer Suppl 72:3515-3524, 1993). The therapy is designed to ease subsequent surgical
35 intervention, increase local control, and improve long-term outcomes of patients. This is partially accomplished by 1) reducing the tumor burden at the primary site, rendering efforts at local control more likely to be completely successful , 2) controlling disease that

is not initially amenable to local treatment, 3) reducing the number of cancerous cells before surgery, and 4) when administered before surgery, less resection may be necessary, thereby potentially sparing normal organ function. The potential disadvantages of neoadjuvant therapy include: acute toxicity of treatment; ineffectiveness which may compromise local control or delay of definitive therapy allowing for aggressive local tumors to metastasize; scarring and fibrosis; making surgery more difficult; retardation of wound healing by decreasing tissue vascularity; and ineffectiveness leading to a higher proportion of quiescent tumor cells.

Despite all of the existing treatments for solid tumors, there still is a substantial unsatisfied need in the field for a more effective method of treating patients, both in terms of efficacy and avoiding the disadvantages of the current methods. The present invention fulfills this need and further provides other related advantages.

Summary of the Invention

It is the object of this invention to cause tumor death *in vivo* by inhibiting blood flow to the tumor by blood clot formation in the vessels that supply blood to the tumor, to inhibit angiogenesis within the tumor by producing inhibitory proteins within the tumor, to produce a protein that activates a non-toxic agent into a toxic form within the tumor, or to produce a protein that competes for or metabolizes nutrients in the blood supply of the tumor.

Within one aspect of the present invention, a method is provided for killing tumor cells *in vivo* comprising transducing cells in or adjacent to a tumor with a recombinant vector comprising a nucleic acid molecule encoding a polypeptide capable of stimulating blood clot formation in or adjacent to the tumor. In various embodiments, the nucleic acid molecules may be selected from the group consisting of Russell's viper venom factor X-activating factor, Russell's viper venom factor V-activating factor, thrombin and thrombin-like enzymes such as those extracted from *Crotalus* adamanteus (Crotalus), *Crotalus* horridus horridus, *Agkistrodon* rhodostoma (Ancrod), *Agistrondon* contortrix contortrix, *Agkristrodon* acutus, *Bothrops* atrox (Batroxobin), *Bothrops* marajoensis, *Bothrops* moojeni, *Trimerersurus* gramineus, *Trimeresurus* okinavensis and *Brirtis* gabonica, tissue factor, truncated tissue factor, cancer procoagulant, and a fusion protein comprising part or all of a procoagulant protein and part or all of a ligand that binds to endothelial cells with high affinity. Within one embodiment of the invention, these nucleic acid molecules encode a protein selected from the group consisting of von Willebrand factor antigen II, endothelial-monocyte-activating polypeptide I, endothelial-monocyte-activating

polypeptide II, tumor necrosis factor α, tumor necrosis factor β, and the tissue factor-inducing factor from *Rickettsia* rickettsii. In still another embodiment, the nucleic acid molecules encode a polypeptide capable of inhibiting fibrinolysis. In certain embodiments, these nucleic acid molecules are selected from the group consisting of α2-antiplasmin, plasminogen activator inhibitor I, plasminogen activator inhibitor II, plasminogen activator inhibitor III, and *Erthrina* proteinase inhibitors.

In another aspect of the invention, a method is provided for inhibiting tumor angiogenesis *in vivo*, comprising transducing cells in or adjacent to the tumor with a recombinant vector comprising a nucleic acid molecule encoding a polypeptide capable of inhibiting vascularization of the tumor. In various embodiments, the polypeptides encoded by the nucleic acid molecule are selected from the group consisting of angiostatin, interferon α, interferon β, platelet factor-4, tissue inhibitors of metalloproteinases I, tissue inhibitors of metalloproteinases II, tissue inhibitors of metalloproteinases III, thrombospondin, the angiogenic fragment of prolactin, heparinase, neutralizing antibody fragments against basic fibroblast growth factor, vascular endothelial cell growth factor and αVβ3 integrin.

In another aspect of the invention, a method is provided for killing tumor cells *in vivo*, comprising transducing cells of a blood vessel in or adjacent to an arterial side of a tumor with a recombinant vector comprising a nucleic acid molecule encoding a polypeptide capable of activating a non-cytotoxic agent capable of being a cytotoxic agent and administering to the animal a non-cytotoxic agent activated by the polypeptide into a cytotoxic agent. Within the various embodiments, nucleic acid molecules encode polypeptides selected from the group consisting of *Herpes* simplex virus thymidine kinase, varicella-zoster virus thymidine kinase, cytosine deaminase, xanthine-guanine phosphoribosyl transferase, *Escherischia* coli purine nucleoside phophorelase, the cytochrome p450 2B1 gene product, alkaline phosphatase, β-glucosidase and nitroreductase.

In yet another aspect of the invention, a method is provided for depriving tumor cells *in vivo* of nutrients, comprising transducing cells in or adjacent to a blood vessel in a tumor with a recombinant vector comprising a nucleic acid molecule encoding a polypeptide capable of binding or metabolizing nutrients in the perivascular interstitial space of the tumor. Within various embodiments, the nucleic acid molecule encoding the polypeptide is selected from the group consisting of soluble folate receptor/folate binding protein, soluble tranferrin receptor, fetal hemaglobin, oxygen-binding fragments of fetal hemoglobin, or a fusion polypeptide consisting of the extracellular matrix binding fragment derived from the β3 integrin and a fragment of a receptor polypeptide selected from the folate receptor or the tranferrin receptor.

In various aspects, the recombinant vectors is a gene delivery vehicle is selected from a recombinant viral vector and a non-viral nucleic acid (DNA or RNA) vector. In a preferred embodiment, the recombinant viral vector is selected from the group consisting of adenovirus, pox virus, poliovirus, rhinovirus, influenza virus, parvovirus, adeno-associated virus, *Herpes* virus, simian virus 40, human immunodeficiency virus, measles virus, astrovirus or corona virus. Preferred viruses are retroviruses and alphaviruses. Particularly preferred retroviruses are avian leukosis virus, bovine leukemia virus, murine leukemia virus, mink-cell focus-inducing virus, murine sarcoma virus, reticuloendotheliosis virus, gibbon ape leukemia virus, Mason-Pfizer leukemia virus or rous sarcoma virus. The most preferred murine retroviruses are Abelson, Friend, Graffi, Gross, Kristen, Harvey sarcoma, raucher, or Moloney leukemia. Particularly preferred alphaviruses are Sindbis virus, Semliki Forest virus, Middleberg virus, Ross River virus Aura virus, Fort Morgan virus or Venezuelan equine encephalitis virus. In addition, in various aspects the recombinant virus is replication defective.

Other aspects of the invention relate to packaging cells, producer cells containing the recombinant viral vectors, viral particles produced from the producer cells, and transduced recombinant viral vector target cells, as well as pharmaceutical compositions comprising vectors according to the invention. Preferred compositions include those comprising lyophilized or dehydrated recombinant virus.

These and other aspects will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1 is a diagramatic representation of the coagulation pathway.

Figure 2 is a schematic illustration of p31N2R5(+).

Figure 3 is a schematic illustration of pN2R3(-).

Figure 5 is a schematic illustration of pN2R3(+).

Figure 6 is a schematic illustration of pN2R5(-).

Figure 7 is a schematic illustration of p31N25V(+).

Figure 8 is a schematic illustration of pTK_A.

Figure 9 is a schematic illustration of pPrTK_A.

Figure 10 is a schematic illustration of pTK-1 and pTK-3.

Figure 11 is a schematic illustration of a SINDBIS Basic Vector and a SINDBIS Luciferase Vector.

Figure 12 is a schematic illustration of a representative embodiment of a Eukaryotic Layered Vector Initiation System.

Figure 13 is a graph which shows a time course for luciferase expression from ELVIS-LUC and SINBV-LUC vectors.

Figure 14 is a schematic illustration of the mechanism for disabling a viral junction region by "RNA loop-out."

Figure 15 is an illustration of one method for modifying a Sindbis junction region.

Figure 16 is a schematic illustration of Sindbis Packaging Expression Cassettes.

Figure 17 is a bar graph which shows SIN-luc vector packaging by representative packaging cell lines.

Figure 18 is a bar graph which shows SIN-luc vector packaging by PCL clone #18 over time.

Figure 19 is a graph which illustrates expression and rescue of a Sindbis-luciferase vector.

Figure 20 is a graph demonstrating retention of viral activity upon reconstitution of a representative recombinant retrovirus lyophilized in a formulation buffer containing mannitol.

Figure 21 is a graph demonstrating retention of viral activity upon reconstitution of a representative recombinant retrovirus lyophilized in a formulation buffer containing lactose.

Figure 22 is a graph demonstrating retention of viral activity upon reconstitution of a representative recombinant retrovirus lyophilized in a formulation buffer containing trehalose.

Figure 23 is a set of representative graphs comparing stability of liquid non-lyophilized recombinant retrovirus stored at -80_C versus lyophilized formulated recombinant retrovirus stored at -20_C, using various saccharides. For ease of comparison, the titers have been normalized.

Figure 24 is a bar graph which illustrates the effect of ganciclovir on CT26, CT26 βgal and CT26TK Neo cells.

Figure 25 is a graph which illustrates the effect of tumor volume over time in a ganciclovir dose study of mice injected with CT26TK Neo.

Figure 26 is a series of four photographs of mice, illustrating the effect of different dose regimens of ganciclovir on intraperitoneal tumor growth.

Figure 27 is a series of four photographs of mice, illustrating the effect of different dose regimens of ganciclovir on subcutaneous tumor growth.

Figure 28 is a schematic illustration of pHCMV-PA.

<u>Definition of Terms</u>

The following terms are used throughout the specification and claims. These terms

5   shall be defined as follows, unless otherwise indicated.

"High Affinity Binding Pair" refers to a set a molecules which is capable of binding one another with a K_D of less than $10^{-y}M$, wherein y is selected from the group consisting of 8, 9, 10, 11, 12, 13, 14 and 15. As utilized herein, the "K_D" refers to the disassociation constant of the reaction $A + B \rightleftharpoons AB$, wherein A and B are members of the high affinity

10   binding pair. (In addition, as is understood by those of ordinary skill in the art, as the affinity of the two molecules increases, K_D decreases.) Disassociation constants may be readily determined by a variety of techniques, including for example by a Scatchard analysis (*see* Scatchard, *Ann. N.Y. Acad. Sci. 51*:660-672, 1949). Representative examples of suitable   affinity   binding   pairs   include   biotin/avidin,   cytostatin/papain,

15   phosphonate/carboxypeptidase A, and 4CABP/RuBisCo.

"Targeting element" refers to a molecule which is capable of specifically interacting with a selected cell type. As utilized within the context of the present invention, targeting elements are considered to specifically bind a selected cell type when a biological effect of the coupled targeting element may be seen in that cell type, or, when there is greater than a

20   10 fold difference, and preferably greater than a 25, 50 or 100 fold difference between the binding of the coupled targeting element to target cells and non-target cells. Generally, it is preferable that the targeting element bind to the selected cell type with a K_D of less than $10^{-5}M$, preferably less than $10^{-6}M$, more preferably less than $10^{-7}M$, and most preferably less than $10^{-8}M$ (as determined by a Scatchard analysis, *see* Scatchard, *Ann. N.Y. Acad.*

25   *Sci. 51*:660-672, 1949). In addition, it is generally preferred that the targeting element bind to the selected cell type with an affinity of at least 1 log (*i.e.*, 10 times) less than the affinity constant of the high affinity binding pair. (In other words, the K_D value will be at least 1 log or 10 fold greater.) Suitable targeting elements are preferably non-immunogenic, not degraded by proteolysis, and not scavenged by the immune system. Particularly preferred

30   targeting elements (which are conjugated to a member of the high affinity binding pair) should have a half-life (in the absence of a clearing agent) within an animal of between 10 minutes and 1 week. Representative examples of suitable targeting elements are set forth below in more detail.

"Clearing agent" refers to molecules which can covalently or non-covalently interact

35   with circulating coupled targeting elements. Preferably, the clearing agent is non-immunogenic, specific to the coupled targeting element, and large enough to avoid rapid renal clearance. In addition, the clearing agent is preferably not degraded by proteolysis,

and not scavenged by the immune system. Particularly preferred clearing agents for use within the present invention include those which bind to the coupled targeting element at a site other than the affinity binding member, and most preferably, which bind in a manner that blocks the binding of the targeting element to its target. Numerous cleaving agents may be utilized within the context of the present invention, including for example those described by Marshall et al. in *Brit. J. Cancer 69*:502-507, 1994.

"Recombinant retroviral vector" refers to an assembly which is, within preferred embodiments of the invention, capable of directing the expression of a sequence(s) or gene(s) of interest. Preferably, the retroviral vector construct should include a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR. A wide variety of heterologous sequences may be included within the vector construct, including for example, sequences which encode a protein (*e.g.*, cytotoxic protein, disease-associated antigen, immune accessory molecule, or replacement protein), or which are useful in and of themselves (*e.g.*, as ribozymes or antisense sequences). Alternatively, the heterologous sequence may merely be a "stuffer" or "filler" sequence of a size sufficient to allow production of retroviral particles containing the RNA genome. Preferably, the heterologous sequence is at least 1, 2, 3, 4, 5, 6, 7 or 8 Kb in length.

The retroviral vector construct may also include transcriptional promoter/enhancer or locus defining element(s), or other elements which control gene expression by means such as alternate splicing, nuclear RNA export, post-translational modification of messenger, or post-transcriptional modification of protein. Optionally, the retroviral vector construct may also include selectable markers that confer resistance of recombinant retroviral vector, transduced or transfected, cells to TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more specific restriction sites and a translation termination sequence.

A "gene delivery vehicle" is a recombinant vehicle, such as a recombinant viral vector, a nucleic acid vector (such as plasmid), a naked nucleic acid molecule such as genes, a nucleic acid molecule complexed to a polycationic molecule capable of neutralizing the negative charge on the nucleic acid molecule and condensing the nucleic acid molecule into a compact molecule, a nucleic acid associated with a liposome (Wang, *et al., PNAS 84*:7851, 1987), a bacterium, and certain eukaryotic cells such as a producer cell, that are capable of delivering a nucleic acid molecule having one or more desirable properties to host cells in an organism. As discussed further below, the desirable properties include the ability to express a desired substance, such as a protein, enzyme, or antibody, and/or the ability to provide a biological activity, which is where the nucleic acid molecule carried by the GDV is itself the active agent without requiring the expression of a desired substance.

- 10 -

One example of such biological activity is gene therapy where the delivered nucleic acid molecule incorporates into a specified gene so as to inactivate the gene and "turn off" the product the gene was making.

"Zymogen" is an inactive enzyme precursor capable of being activated to a catalytic state by post-translational modification. Briefly, some zymogens contain a polypeptide chain which blocks enzyme utility. The enzyme is activated by acid or enzymatic cleavage that removes the inhibitory polypeptide chain.

Detailed Description of the Invention

This invention provides several methods for gene therapy-mediated treatment of solid tumors with recombinant vectors based on interference with nutrient supply to the tumor cells caused by (i) selectively initiating irreversible coagulation of the tumor vasculature by transferring a gene for any one of several potent diffusible procoagulant proteins to intratumoral vascular endothelial cells or perivascular tumor cells, (ii) inhibition of tumor neovascularization by transfer of a gene encoding an anti-angiogenic protein to intratumoral vascular endothelial cells or perivascular tumor cells, (iii) destruction of the tumor vascular bed by selective killing of intratumoral vascular endothelial cells by transfer of a 'suicide' gene and (iv) starving the tumor by creating a microenvironment in perivascular areas which absorbs or metabolizes vital incoming nutrients, accomplished by transfer of genes encoding soluble extracellular matrix-binding nutrient receptors or specific catabolic enzymes to perivascular tumor cells.

Within one aspect of the invention a method is provided for selectively initiating or exacerbating coagulation of the tumor vasculature by transferring any of a number of genes for procoagulant proteins, fusion proteins comprising at least a procoagulant portion of a procoagulant protein and at least that portion of a ligand which binds with high affinity to endothelial cells, inducers of procoagulant proteins or inhibitors of fibrinolysis to intratumoral vascular endothelial cells or perivascular tumor cells using a gene delivery vehicle according to the invention.

The establishment of a critical hypercoagulative state in the tumor vascular bed can be achieved by inducing local secretion of three broad classes of proteins. The first group comprises procoagulant proteins (e.g., Russell's viper venom factor X-activating factor, Russell's viper venom factor V-activating factor, tissue factor, truncated tissue factor, cancer procoagulant, thrombin, thrombin-like enzymes, and fusion proteins comprising all or at least the extracellular portion of a procoagulant protein operatively fused to all or at least that portion of a ligand which binds with high affinity to an endothelial cell membrane

or protein associated therewith) that directly activate one or more of the zymogens that make up the physiologic clotting cascade. The second group comprises proteins produced by tumor cells, leukocytes and certain infectious organisms (*e.g.*, von Willebrand factor santigen II, endothelial-monocyte-activating polypeptide I, endothelial-monocyte-activating

5   polypeptide II, tumor necrosis factor α, tumor necrosis factor β, and the tissue factor-inducing factor from *Rickettsia* rickettsii) that induce the expression of a key procoagulant protein, such as tissue factor, by host endothelial cells and monocytes/macrophages, indirectly resulting in local activation of the clotting cascade. The third group of proteins inhibits the homeostatic response to coagulation (*i.e.*, fibrinolysis), *e.g.*, α2-antiplasmin,

10 plasminogen activator inhibitor I, plasminogen activator inhibitor II, plasminogen activator inhibitor III, and *Erythrina* proteinase inhibitors. Members of the third group of proteins work in concert with procoagulant proteins in inducing local blood clotting or, in tumors which are themselves procoagulant in nature, tip the balance between fibrin production and degradation resulting in coagulation of the tumor vascular bed in the absence of exogenous

15 procoagulant activity.

The pivotal point in the clotting cascade, where the intrinsic and extrinsic pathways converge, is the activation of Factor X (FX) to FXa (*see* Figure 1). In the extrinsic pathway, tissue factor (TF) activates FVII to FVIIa and then combines with FVIIa in the endothelial cell membrane to activate FX to FXa. In the intrinsic pathway, FXII is

20 activated to FXIIa as a result of tissue injury or exposure to air and FXIIa in turn activates FXI to FXIa which then converts FVIII and FIX on the surface of perturbed endothelial cells or platelets to their respective active forms. FX is activated to FXa by FVIIIa/FIXa complexes in the presence of calcium ions and a phospholipid membrane (*see* Figure 1). In the subsequent common pathway, a complex of FXa with FVa in the endothelial or platelet

25 membrane with Ca^{2+} ions forms the prothrombinase complex, which efficiently catalyses the conversion of prothrombin to thrombin. The prothrombinase complex will generate 1,500 moles(m)/minute(m/min) of thrombin/mole of FXa. Thrombin degrades plasma fibrinogen to fibrin and activates FXIII to FXIIIa which, in turn, cross-links the fibrin molecules to form an insoluble matrix (*see*Figure 1).

30 Procoagulant factors can act at several points in the clotting cascade. Tissue factor (TF) and truncated TF combine with FVIIa and a phospholipid bilayer to activate FX (Nemerson, *et al.*, *Prog. Hemost. Thromb.* 6:237, 1982). Russell's Viper venom FX-activating factor (RVV-X) and cancer procoagulant (CP) activate FX directly, independently of TF, FVII, FVIII, or FIX (Gordon, *et al.*, *Hemat. Onco. Clinics North*

35 *America* 6:1359, 1992; Takeya, *et al.*, *J. Biol. Chem.* 267:14109, 1992). Thrombin and the wide range of thrombin-like enzymes that have been isolated from the venoms of numerous snakes (Doull, *et al.*, *Toxicology*. eds. MacMillan Pub. Co., NY 1986), for example,

Crotalus adamanteus (Crotalase), *Crotalus* horridus horridus, *Agkistrodon* Rhodostoma (Ancrod), *Agkistrodon* contortrix contortrix, *Agkistrodon* acutus, *Bothrops* atrox (Batroxobin), *Bothrops* marajoensis, *Bothrops* moojeni, *Trimeresurus* gramineus, *Trimeresurus* okinavensis and *Bitis* gabonica, all of which degrade fibrinogen directly and

5   thus are independent of most of the clotting cascade. TF, RVV-X, and CP are extremely potent inducers of coagulation because their effects are amplified by subsequent stages of the zymogen cascade. Other inducers of coagulation include Russell's viper venom factor V-activating factor and truncated tissue factor. Thrombin and thrombin-like enzymes require fibrinogen and FXIII to cause clotting. Many tumors induce local fibrin formation

10 as a part of the malignant process (Flier, *et al., New Eng. J. Med. 315*:1650, 1986) and it is thought that the clotting factors detected immunohistochemically in the tumor interstitium (Zacharski, *et al., Cancer 60*:2675, 1987) leak out as a result of tumor-induced hyperpermeability of local blood vessels (Senger, *et al., Science 219*:993, 1983). TF targeting has been shown to be effective in one tumor model (Moloma, *et al.* submitted for

15 publication). As such, at present the use of early-acting procoagulants, such as TF, CP and RVV-X, is preferred.

In the last century, Coley reported that several late-stage cancer patients injected with a live bacterial preparation developed severe septicemia but subsequently recovered and were cured of their tumors (Coley, *et al., Am. J. Med. Sci. 105*:487, 1983). This

20 activity was eventually found to be mediated by a cytokine released by macrophages in response to bacterial endotoxin that caused specific hemorrhagic necrosis in Meth-A fibrosarcomas in mice and so was called tumor necrosis factor (TNF, Old, *et al., Science 230*:630, 1986; Carswell, *et al., PNAS 72*:3666, 1975; Nawroth, *et al., J. Exp. Med. 168*:637, 1988). When TNF infusions failed to show similar efficacy in other animal

25 models and clincial trials (Kao, *et al., Behring Inst. Mitt. 92*:92, 1993), it was reasoned that unique synergistic cofactors were produced by Meth-A cells that sensitized the tumors to TNF. Two such factors, endothelial-monocyte-activating polypeptides I and II (EMAP-I, EMAP-II), have recently been isolated and cloned (Kao, *et al., supra;* Kao, *et al., J. Biol. Chem. 269*:9774, 1994). TNF and EMAP-I and II all induce weak tissue factor activity in

30 endothelial cells and monocytes but act synergistically when combined (Kao, *et al., supra;* Clauss, *et al., J. Biol. Chem. 265*:7078, 1990). This syngergistic effect may be utilized for procoagulant gene therapy of solid tumors. Firstly, the EMAP-I or -II genes (Kao, *et al., Behring Inst. Mitt. 92*:92, 1993; Kao, *et al., J. Biol. Chem. 269*:9774, 1994) may be combined with a TNF gene to produce potent bicistronic procoagulant gene therapy

35 vectors. Secondly, increased specificity of procoagulant effects may be produced by introducing EMAP-I or -II, TNF (TNFα or β), or tissue factor-inducing factor from *Rickettsia* rickettsii genes into separate vectors which are selectively targeted to tumor cells

or tumor endothelial cells by targeting vehicles (*e.g.* antibodies or other cell/tissue specific ligands, proliferation-dependent retroviral vectors) that have few or no common cross-reactivities. Thirdly, EMAPs I or II selectively generated at the tumor site by vectors may be combined with systemic therapy with recombinant TNF or flavone acetic acid (FAA), a low molecular weight antitumor agent that is effective against Meth-A tumors but less so in other systems (Murray, *et al.*, *Int. J. Cancer 49*:254, 1991). Another procoagulant protein that may be utilized in the context of this invention is von Willebrand factor antigen II.

Disruption of the balance between fibrin formation and fibrinolysis may further promote clot formation. Specifically, many animal and human tumors are intrinsically prothrombotic (Flier, *et al. supra.*) as a result of local vessel disruption or tumor cell production of TF, TF-like activity, or cancer procoagulant (Gordon, *et al.*, *supra.*). This process may account, at least in part, for the areas of necrosis in solid tumors and it is thought that functional vascular integrity sufficient to support tumor growth is maintained *in vivo* by local fibrinolysis that largely keeps pace with fibrin formation (Dvorak, *et al.*, *Cancer Res. 44*:3348, 1984). Fibrin is degraded by the protease plasmin, which is generated from the zymogen plasminogen by the action of plasminogen activators (Dane, *et al.*, *Adv. Cancer Res. 44*:139, 1985). Both tissue-type (T-PA) and urokinase-type (U-PA) plasminogen activators are secreted by endothelial cells and tumor cells (Dane, *et al.*, *supra.*). Transfer of genes encoding T-PA or U-PA antagonists to intratumoral endothelial cells or perivascular tumor cells would result in decreased plasmin levels. Several candidate proteins could also perform this function if delivered to tumors by recombinant vectors. Plasminogen activator inhibitors I, II, and III (PAI-I, PAI-II, and PAI-III) are competitive inhibitors of plasminogen activators that bind to T-PA and U-PA reversibly, blocking plasminogen cleavage (Wun, *et al.*, *Crit. Rev. Biotech. 8*:131, 1988). $\alpha2$-antiplasmin is the major plasmin inhibitor in the blood, where it is constitutively present at fairly low levels, irreversibly inactivating plasmin until plasmin becomes present in excess (Aoki, *et al.*, *Semin. Thromb. Hemostasis 10*:24, 1984). Several specific protease inhibitors have been cloned from the legumes *Erythrina* caffra and *Erythrina* latissima (Teixeira, *et al.*, *Biochem. Biophys. 1217*:23, 1994). These proteins have extensive homology to soybean trypsin inhibitor and are potent inhibitors of plasmin and T-PA (Teixeira, *et al.*, *Biochem. Biophys. 1217*:23, 1994). In addition to promoting coagulation of the tumor vascular bed, plasmin antagonists such as $\alpha2$ anti-plasmin, have additional antitumor activities. Overexpression of U-PA by tumor cells is associated with increased metastatic potential and angiogenic activity (Meissauer, *et al.*, *Exp. Cell Res. 192*:453, 1991). U-PA generates plasmin which, in addition to degrading fibrin, also cleaves procollagenase to collagenase and releases bioactive angiogenic growth factor from the tumor's extracellular matrix. Collagenase is thought to contribute to the degradation of

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interstitial stroma and basement membranes that is essential to (i) invasion of tumor cells into surrounding tissues (ii) intravasation into local blood vessels and escape from the primary tumor site and (iii) migration of endothelial cells during neovascularization (Goodson, *et al.*, *PNAS 91*:7129, 1994). Thus, inhibition of plasmin production or activity
5   impacts upon the pathogenesis of malignancy at several levels.

One embodiment of the present invention provides for the expression of genes for procoagulant or antifibrinolytic proteins in capillary endothelial cells (EC) lining the vessels of the tumor vascular bed, or in the subpopulation of tumor cells closest to these vessels. Expression of the gene(s) coding for procoagulant or antifibrinolytic proteins from these
10   'perivascular' tumor cells (PTC) is important because such expression will provide concentrations of plasma-derived clotting factors sufficient to cause clot formation, and because the coagulation process initiated at the tumor cell surface will spread 'back' to nearby vessels to induce widespread ischemia of the tumor. In a prefered embodiment, a gene delivery vehicle containing a nucleic acid molecule encoding a procoagulant or
15   antifibrinolytic protein is targeted to perivascular tumor cells, as described below.

In another preferred embodiment of this approach, the tropism of a gene delivery vehicle is combined with the specificity of a polypeptide encoded thereby to achieve operative tumor vascular specificity. For example, a targeted or untargeted gene delivery vehicle can be used to deliver to perivascular tumor cells a recombinant expression vector
20   carrying a fusion gene which encodes a protein having procoagulant activity and tissue specificity. In one instance, the fusion gene encodes a fusion protein comprising at least the domain(s) responsible for procoagulant activity of a procoagulant protein (*e.g.*, tissue factor, truncated tissue factor Russell's Viper Venom Factor X-activating factor, and cancer procoagulant) fused to at least the receptor binding domain(s) of a ligand which binds with
25   high affinity to a receptor present on an endothelial cell membrane. Following expression in perivascular tumor cells, the fusion protein is secreted into the perivasular space. After diffusion to nearby endothelial cells of the tumor vascular, receptors bind to the ligand portion of the fusion protein to produce a membrane-bound multimolecular array capable of initiating thrombus formation on the lumenal surface of endothelial cells of the tumor
30   vascular bed.

Within a particularly preferred embodiment, the fusion protein comprises truncated tissue factor (lacking the transmembrane and intracellular domains; Ruf, *et al.* (1994), *J. Biol. Chem.*, vol. 266:2158) at its amino terminus fused by way of a flexible oligopeptide spacer to the receptor binding domains of VEGF [Peretz, *et al.* (1992), *Biochem. Biophys*
35   *Res. Commun.*, vol. 182, *no.* 3:1340-1347], the receptor for which is overexpressed in endothelial cells. The gene encoding this fusion protein (tTF-VEGF) is assembled using standard recombinant techniques. Other preferred fusion proteins comprise Russell's Viper

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Venom Factor X-activating factor or cancer procoagulant in place of tTF, although many other proteins or portions thereof can also be used. In addition, receptor binding domains from ligands which bind with high affinity to receptors on endothelial cells may be substituted for the receptor binding domains of VEGF. Such ligands include, but are not

5   limited to, TGF-β, FGF, PDGF, u-PA, u-PA receptor antagonist, and ligands for endoglin, endosialin, CD31, CD34, integrin αVβ3, and antigen binding fragments of monoclonal antibodies reactive against receptors found on endothelial cells.

The invention further provides methods for inhibiting tumor growth or causing regression of established tumors by inhibiting tumor angiogenesis via vector-mediated

10   transfer of genes encoding anti-angiogenic polypeptides.

The growth of solid tumors beyond a diameter of 1-2 mm is dependent upon the recruitment of new blood vessels growing into the tumor from surrounding host tissue (Folkman, et al., J. Nat. Cancer Inst. 82:4, 1990; Folkman, et al., N. Engl. J. Med. 285:1182, 1971). A critical step in malignant progression is the acquisition of angiogenic

15   activity by tumor cells (Folkman, et al., Nature 339:58, 1989). The uncontrolled growth of tumor cells in a confined space often causes physical collapse of intratumoral capillaries. Blocking of angiogenesis may result in an acute hypovascular state and lead to actual tumor regression, rather than just inhibition of growth (Ingber, et al., Nature 348:555, 1990). Numerous proteins secreted by tumor cells have been shown to exert angiogenic activity in

20   several in vivo assays (Bicknell, et al., Eur. J. Cancer 27:785, 1991), but two cytokines, basic fibroblast growth factor (bFGF) and vascular endothelial cell growth factor (VEGF) have been proven to function as tumor angiogenesis factors in situ, because transduction with bFGF or VEGF cDNA confers tumorigeneity on non-tumorgenic cell lines (Winer, et al., supra) and blocking of cytokine activity with specific monoclonal antibodies inhibits

25   tumor growth and vascularization (Kim, et al., supra).

Angiogenesis is a complex process involving at least four steps (Folkman, eds. J.B. Lippincott, Philadelphia, pp. 42-62, 1985). Host endothelial cells (EC) (i) degrade their underlying basement membrane (ii) migrate towards the angiogenic stimulus, (iii) proliferate to seed new capillary buds and (iv) anastamatose with an existing vessel, lay

30   down a new basement membrane, and initiate blood flow through the new capillary loop (Folkman, eds. J.B. Lippincott, Philadelphia, pp. 42-62, 1985). Since each step is essential to the process, inhibitors of angiogenesis may exert their effects in a variety of ways. Physiological angiogenesis, such as in wound healing, is regulated by a balance between angiogenic and anti-angiogenic factors (Fidler, et al. Cell 79:185, 1994). To date, at least

35   ten anti-angiogenic proteins have been described (Fidler, et al., supra; O'Reilly, et al., Cell 79:315, 1994; Ratinejad, et al., Cell 56:345, 1989; Moses, et al., J. Cell. Biochem. 47:230, 1991; and Bicknell, et al., supra): including angiostatin, α-IFN, β-IFN, platelet factor-4,

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tissue inhibitor of metalloproteinases (TIMP-I, TIMP-II, TIMP-III), and glioma-derived angiogenesis inhibiting factor (Van Meir, *et al.*, *Nature Genetics* 8:171, 1994), thrombospondin, the anti-angiogenic fragment of prolactin, heparinase or other appropriate peptide proteins or poly nucleotides that directly or indirectly (*e.g.*, by catalysis of
5 production of inhibitors such as retinoic acid or its analogs) inhibit cell migration, endothelial cell proliferation, basement membrane degradation or 3-D organization of new blood vessels (Auerbach, W. and Auerbach, R. *Pharmac. Ther.*, 63:265, 1994). Blocking monoclonal antibodies against bFGF, VEGF, and the $\alpha V\beta_3$ integrin are also angiogenesis inhibitors. α-IFN has been shown to induce complete regression of life-threatening
10 hemangiomas in infants and highly vascular Kaposis sarcoma (Ezekowitz, *et al.*, *N. Engl. J. Med.* 326:1456, 1992; Real, *et al.*, *J. Clin. Oncol.* 4:544, 1986).

The present invention provides for the use of any one of the physiological polypeptide negative regulators of angiogenesis in anti-angiogenic strategies where the gene encoding the anti-angiogenic factor is delivered to either tumor endothelial cells or
15 perivascular tumor cells using a recombinant vector. For example, α-IFN downregulates bFGF transcription and translation (Singh, *et al.*, *Am. J. Pathol.* 145:365, 1994), consequently, it is most effective if produced within tumor cells. TIMPs inhibit endothelial cell proteases required for extracellular matrix degradation during EC migration (Moses, *et al.*, *supra*) and therefore would be active in solution anywhere in the tumor interstitium.
20 Thrombospondin or blocking anti-FGF or anti-VEGF antibodies antagonize angiogenic cytokine activity at the endothelial cell surface (Kim, *et al.*, *supra*; Fidler, *et al.*, *supra*) and are most effective if expressed in the vasculature itself.

Anti-angiogenic polypeptide genes may be delivered selectively to perivascular tumor cells with a second generation antibody-targeted recombinant vector. Suitable tumor
25 markers for targeting include c-erbB2/p185[HER-2] (Shepard, *et al.*, *supra*), TAG-72 (Guadagni, *et al.*, *supra*), carcinoembryonic antigen (CEA, Guadagni, *et al.*, *supra*), the high affinity folate receptor (Bottero, *et al.*, *supra*), polymorphic epithelial mucin (PEM, Rettig, *et al.*, *supra*), epidermal growth factor receptor (Rettig, *et al.*, *supra*), a CD44 isoform (Rettig, *et al.*, *supra*) and prostate-specific antigen (Mulders, *et al.*, *supra*).
30 Another approach is to deliver angiostatin *ex vivo*. Angiostatin is produced by cells in primary tumor masses but suppresses neovascularization of distant metastases, perhaps explaining the clinical observation that metastases grow more quickly in some patients after the primary tumor is resected (O'Reilly, *et al.*, *supra*). Thus, angiostatin is sufficiently potent and stable in the circulation to exert endocrine effects, so it may be possible to
35 mimic the presence of the primary tumor by transplantation of a population of normal long-lived non-tumorigenic cells that have been stably transduced with recombinant vector containing an angiostatin gene. In addition, direct administration of a vector encoding

angiostatin may lead to sufficient local or systemic levels to inhibit growth of primary tumors or metastases.

Because of their proven efficacy in the clinic or in animal models, α-IFN, TIMP-1 or angiostatin are particularly preferred candidates for anti-angiogenic gene therapy, however recombinant single-chain Fv genes from anti-VEGF antibodies are also preferred.

The present invention further provides a method for selectively killing tumor endothelial cells with 'suicide' gene therapy. In general, the physiologic activation of the clotting cascade is a mechanism to stop blood flow and seal the damaged tissue from exposure to the environment. Consequently, damage to vascular endothelial cells that line blood vessels is an initiator of both the intrinsic and extrinsic pathways of coagulation (Kao, et al., supra). Retraction of damaged endothelial cells from the underlying subendothelial matrix exposes procoagulant factors to the blood. Tissue factor in the subendothelial space causes activation of FX by the extrinsic pathway (Drake, et al., Am J. Pathol. 134:1087, 1989) and platelets that adhere to and become activated by matrix proteins intitiate the intrinsic pathway (Stern, et al., Haemostasis 18:202, 1988). Thus, therapeutic strategies that result in the death of significant numbers of endothelial cells in the tumor vascular bed may produce a similar result, particularly, thrombosis and ischemic necrosis of the tumor parenchyma. Recently, this was demonstrated in a murine model of a solid tumor therapy via killing of tumor endothelial cells. A mouse tumor line was transduced with a retroviral vector containing a murine γ-IFN gene. When the transduced tumor cells were grown in nude mice, they secreted γ-IFN which activated local endothelial cells (EC) in a paracrine fashion to express MHC Class II antigens (Burrows, et al., Cancer Res. 52:5954, 1992). Since normal EC are Class II-negative, anti-Class II antibodies were utilized that bound specifically to tumor EC in this model (Burrows, et al., Cancer Res. 52:5954, 1992). An immunotoxin constructed by chemically conjugating ricin A-chain to the anti-Class II monoclonal antibody produced dramatic regressions of large (1 cm diameter) solid tumors (Burrows, et al., PNAS 90:8996, 1993). A time course study after treatment with the anti-tumor endothelial cell immunotoxin demonstrated that necrosis of the tumor mass was secondary to classical intravascular thrombosis. Denudation of dead endothelial cells from the subendothelial matrix was rapidly followed by focal adhesion and clumping of activated platelets, forming an immature platelet/red cell-rich clot with subsequent fibrin cross-linking producing a permanent, fibrous thrombus (Burrows, et al., J. Controlled Release 28:195, 1994). All these events were complete within 4 hours after injection of the immunotoxin, but early necrotic changes in tumor cells were not observed until 6 hours, indicating that the tumor cells were killed as a result of nutrient deprivation following cessation of blood flow (Burrows, et al., PNAS 90:8996, 1993).

An effective method of killing tumor endothelial cells is to transfer 'suicide genes'
into the tumor vascular wall using gene delivery vehicles followed by intravenous delivery
of a prodrug that is activated by the transgene product within the cytoplasm of the tumor
endothelial cell to produce a cytotoxic agent (Moolten, *et al.*, *Cancer Gene Therap.* *1*:279,
5   1994). A number of alternative 'suicide genes' may be useful for cancer gene therapy
(Moolten, *et al.*, *supra*). Introduction of the bacterial cytosine deaminase gene (Huber, *et
al.*, *Cancer Res.* *53*:4619, 1993) into tumor cells confers sensitivity to the antifungal agent
5-fluorocytosine (5-FC). Cytosine deaminase converts 5-FC to 5-fluorouracil (5-FU,
Nishiyama, *et al.*, *Cancer Res.* *45*:1753, 1985). Since 5-FU is commonly used
10 chemotherapeutic drug for breast cancer, several groups have developed cytosine
deaminase-based 'suicide gene' therapy models for this disease. Tumor specificity may be
further increased by introducing the c-erbB2 promoter/enhancer elements 5' to the cytosine
deaminase gene, so that the therapeutic transgene is preferentially transcribed in c-erbB2-
overexpressing breast tumor cells (Harris, *et al.*, *Gene Therap.* *1*:170, 1994). Alkaline
15 phosphatase has been widely explored as prodrug-activating enzyme in the related field of
antibody directed enzymne-prodrug therapy (ADEPT). This enzyme has the advantage that
it can activate a wide range of phosphorylated derivatives of anticancer agents (*e.g.*
mitomycin C, etoposide, *etc.*) that cannot cross cell membranes until the charged phosphate
group is cleaved off, so a single enzyme could generate *de novo* a cocktail of
20 chemotherapeutic agents within the tumor mass (Senter, *et al.*, *Bioconjugate Chem.* *4*:3,
1993). Other suicide genes may encode a polypeptide or polypeptides (with a
corresponding non-cytotoxic agent) such as Herpes Simpex virus thymidine kinase
(gancyclovir or acyclovir), Varicella Zoster virus thymidine kinase (6 methoxypurine
arabino nucleoside; Huber, *et al.*, *PNAS* *88*:8039, 1991), *E. coli* cytosine deaminase
25 (fluorouracil; Mullen, C.A., *et al.*, *PNAS USA* *89*:33, 1992), *E. coli* xanthine-guanine
phophoribosyl transferase (thioxanthine; Beshard, C., *et al.*, *Mol. Cell Biol.* *7*:4139, 1987),
E. coli or Leishmania purine nucleotide phosphorylase (various nontoxic purine
deoxyadenosine, adenosine, deoxyguanosine, or guanosine derivaties (Koszalka, G. and
Krenitsky, T.A., *J. Biol Chem* *254*:8185, 1979; Sorscher, E.J., *et al.*, *Gene Therapy*, *1*:233,
30 1994), cytochrome pla50 2B1 or cytochrome p450 reductase (*e.g.*, 3amino-1,2,4
benzotriazine 1,4-dioxide (Walton, M.I., *et al.*, *Biochem. Pharmacol.*, *44*:251, 1992), cell
surface alkaline phosphatase (*e.g.*, etoposide monophosphate; Senter, P.D., *et al.*, *PNAS
USA*, *85*:4842, 1988), nitroreductase (*e.g.*, metronidazole or nitrofurantoin; Hof, H, *et al.*,
Immunitat und Infektion, *16*:220, 1988), N-deoxyribosy transferase (1-deazapurine;
35 Betbeder, D., *et al.*, *Nucleic Acids Res* *17*:4217, 1989), pyruvate ferrodoxin
oxidoreductase (metronidazol; Upcroft, J.A., *et al.*, *Int. J. Parasitology*, *20*:489, 1990),
carboxypepidase G2 (aminoacylate nitrogen mustards; Antoniw, P., *et al.*, *Brit J. Cancer*

62:909, 1990), carboxypeptidase A (methotrexate alpha alanine; Haenseler, E., *et al.*, *Biochemistry 31:891*, 1992), β lactamase (cephalosporin derivatives; Meyer, D.L., *et al.*, *Cancer Res. 53*:3956, 1993; and Vradhula, V.M., *et al.*, *Bioconjugate Chemistry 4*:334, 1993), Actinomycin D synthetase complex (synthetic pentapeptide lactone precursors;
5 Katz, E., *et al.*, *J. Antibiotics 43*:231, 1990), and β-glucuronidase (various glucuronide derivatives of toxic drugs such as doxorubicin; Bosslet, K., *et al.*, *Cancer Res. 54*:2151, 1994; Haeberlin, B., *et al.*, *Pharmaceutical Res. 10*:1553, 1993).

The HSV-TK system has important advantages for anti-tumor endothelial cell therapy. In particular, HSV-TK transduced tumor cells can mediate a significant bystander
10 killing effect on untransduced neighboring cells *in vitro* and *in vivo* (Moolten, *et al.*, *supra*; Freeman, *et al.*, *Cancer Res. 53*:5274, 1993), most commonly as a result of transfer to the toxic ganciclovir metabolite, GCV triphosphate, between adjacent cells through intercellular gap junctions (Bi, *et al.*, *Human Gene Therap.*, *4*:725, 1993). Endothelial cells in capillary walls are connected by gap junctions, so a dramatic bystander effect created by
15 GCV-triphosphate transfer between neighboring endothelial cells and the massive amplification effects of the clotting cascade and the tumor to endothelial cell ratio (Burrows, *et al.*, *supra*; Denekamp, *et al.*, *Cancer Topics 6*:6, 1986; Denekamp, *et al.*, *Prog. Appl. Microcir. 4*:28, 1984)) could ensue. Recent evidence suggests that the occasional transduction of tumor endothelial cells during intralesional therapy with HSV-
20 TK retroviral vectors may account for a significant component of the antitumor activity of the vectors (Ram, *et al.*, *J. Neurosurg. 81*:256, 1994). In addition, the suicide gene is only conditionally cytotoxic to the target cells (*i.e.* only when GCV is given). Consequently, an *ex vivo* administration method may be utilized. Briefly, endothelial cells may be isolated from tumor biopsies (Medzelewski, *et al.*, *Cancer Res. 54*:336, 1994), induced to
25 proliferate with appropriate mitogens (Ferrara, *et al.*, *supra*) and transduced with TK *in vitro*. Transplanted EC become incorporated into the neovasculature in days to weeks after intratumoral injection (Lal, *et al.*, *Cancer Gene Therap. 1*:322, 1994), so GCV treatment would follow a suitable 'lag phase' to allow the transduced EC to integrate functionally in to the tumor vasculature. This approach circumvents the requirement for vectors to target the
30 tumor endothelium after intravenous injection. Finally, the two-step enzyme-prodrug system offers greater flexibility of delicate clinical management, because cessation of GCV infusion in the event of (potentially very serious) complications arising from damage to normal EC, would block toxicity without the need to block transgene activity *in situ*.

This invention also provides methods for starving tumor cells of specific nutrients
35 that are required to sustain the hyperactive metabolism and uncontrolled proliferation that characterizes the malignant state. Briefly, recombinant vectors are introduced into

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perivascular tumor cells which contain gene sequences of competitive inhibitors of nutrient receptors or specific nutrient-degrading enzymes.

Oxygen and all other nutrients that are required for tumor cell growth and survival which are generated by catabolic metabolism in the gut and liver enter established tumor masses through their blood supply. Selective expression of proteins that bind or degrade incoming nutrients in the perivascular interstitial space rapidly starve distal tumor cells by nutrient deprivation.

Tumor growth is clearly rate-limited by oxygen availability because the classic 'cord' structure of tumor cells (and many normal cells) around a central vessel is defined in radius by the diffusion distance of oxygen (Denekamp, et al., Cancer Topics 6:6, 1986). Diffusion of oxygen can be retarded in the perivascular interstitial space of the tumor by therapeutic gene transfer and expression of fetal hemoglobin, or oxygen-binding fragments of fetal hemoglobin.

Other methods of decreasing the available oxygen are to deliver the genes for enzymes that produce molecules capable of binding and displacing the oxygen molecules in the oxygenated hemoglobin complex. Such enzymes include those that synthesize cyanide (e.g., Wissing F., et al., J. Bacteriol 121:695, 1975; Cutler, A.J., et al., Arch. Biochem Biophys, 238:272, 1985) a carbon monoxide (heme oxygenases; Murphy, B.J., et al., Cancer Res. 53:2700, 1993)

Reduced forms of folic acid are an essential vitamin required by cells to synthesize nucleotide bases and perform one-carbon transfer reactions (Kamen, et al., PNAS 83:5983, 1986). Many tumor cells overexpress a high-affinity folate receptor (see below) and are partially or completely growth-inhibited by folate deprivation (Matsue, et al., PNAS 89:6006, 1992). A family of chemotherapeutic drugs that includes methotrexate exert their antitumor activity by antagonism of the folate pathway (Koong-Nah, et al., J. Clin. Invest. 91:1289, 1993). A truncated soluble form of the folate binding protein/folate receptor has been cloned (Weltman, et al., Cancer Res. 52:3396, 1992), which competes with cellular integral membrane receptors if overexpressed in perivascular tumor cells as a result of transfer of the gene with a recombinant vector. Another polypeptide capable of binding nutrients in the perivascular interstitial space of the tumor that may be used is soluble transferrin receptors.

It is possible that expression of simple stoichiometric binding proteins in the perivascular tumor interstitium may be overcome by excess nutrients entering the tumor mass. This could be ameliorated by transferring genes that degrade or render resistant to cellular uptake key nutrients, such as folate (Huennekens, F., et al., NCI Monographs 5:1, 1987; Huennekens, F., et al., Advances in Enzymol and Related Areas of Mol. Biol. 47:313, 1978), glucose (hexokinase), NAD (NAD phosphorylase), bases or nucleotides

(adenase, nucleotide hydrolases, or uricase; Wu, X., *et al.*, *PNAS USA 91*:742, 1994; Leyoux, R., *et al.*, *J. Biol. Chem. 267*:8565, 1992; Ito, M., *et al.*, *Genomics 11*:905, 1991)

To prevent possible diffusion of soluble nutrient receptor-binding proteins or degradative enzymes into the surrounding tissue, these genes may be engineered to contain DNA sequences encoding binding domains for extracellular matrix proteins. Such fusion, or "chimeric," proteins would be secreted by perivascular tumor cells and then would be rapidly bound to the plentiful extracellular matrix proteins, including collagens, laminin, fibronectin, vitronectin and von Willebrands factor (vWF), that are part of the tumor stroma and subendothelial basement membrane. The integrins are a superfamily of heterodimeric adhesion proteins expressed by all cells. In ectoderm-derived tissues (such as epithelial and endothelial cells) integrin function to anchor cells to each other and to tissue stromal elements, directing tissue organization and regulating cell growth and differentiation (Hynes, *et al.*, *Cell 69*:11, 1992). Many integrins, particularly those of the ß1 and ß3 families, bind specifically to extracellular matrix (ECM) proteins, often through recognition of a common tripeptide, Arg-Gly-Asp (RGD), present in collagen, fibronectin, vitronectin and vWF (Ruoslahti, *et al.*, *Science 238*:491, 1987). Inclusion of an RGD recognition sequence from one of these integrins in a recombinant nutrient receptor or enzyme gene should permit sequestration of the therapeutic protein in the locale required for optimal activity. The RGD recognition site in the ß3 integrin has been isolated and sequenced (Fitzgerald, *et al.*, *J. Biol. Chem. 262*:3936, 1987). This domain may be included in ECM-binding nutrient receptor antagonists since it recognizes a number of different ECM proteins. Particularly preferred fusion proteins are those comprising an extracellular matrix binding fragment derived from ß3 integrin and a fragment of the folate or transferrin receptors.

One way of targeting vectors to tumor endothelial cells in animals may be achieved using a chimeric envelope protein that has been engineered to contain an avidin domain (that binds specifically to biotin) and then complexed with biotinyl derivatives of any of several monoclonal antibodies (MAbs) that are selective or specific for capillary endothelial cells in solid tumors. Several such reagents have been described in the literature (Burrows, *et al.*, *Pharmac. Ther. 64*:155, 1994). For example, such reagents include MAb FB5, recognizes a heavily sialylated glycoprotein, endosialin, that is expressed at low to moderate levels by tumor endothelial cells and reactive fibroblasts but is absent from the vasculature of normal tissues (Rettig, *et al.*, *PNAS 89*:10832, 1989); TEC-4 and TEC-11, which react with endoglin (CD105), a Type III TGFß receptor that is upregulated; and LM609, a MAb that recognizes a conformational determinant formed by epitopes from the α and ß chains of the human vitronectin receptor (integrin αvß3, CD51/CD61). In samples of normal and malignant human skin tissue, LM609 stained vessels only in the tumor

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sample (Brooks, *et al., Science 264*:569, 1994). The αvβ$_3$ integrin appears to be involved in neovascularization since LM609 inhibited angiogenesis in several models (Brooks, *et al., supra*). One of the more specific and broadly applicable tumor endothelial cell markers is the receptor-bound vascular endothelial cell growth factor (VEGF). VEGF is a potent EC

5   mitogen that is implicated in tumor angiogenesis in many models (Winer, *et al., J. Clin. Invest. 91*:160, 1993; Kim, *et al., Nature 362*:841, 1993). This cytokine accumulates specifically in the tumor vasculature (Dvorak, *et al., J. Exp. Med. 174*:1275, 1991), even though neither VEGF synthesis nor VEGF receptor expression are specfic to tumors (Jakeman, *et al., J. Clin. Invest. 89*:244, 1992; Ferrara, *et al., J. Cell Biochem. 47*:211,

10   1991). MAbs have been raised against non-receptor-binding epitopes of human and guinea-pig VEGF (Thorpe, *et al. unpublished*). When injected into nude mice bearing human tumor xenografts or tumor-bearing guinea pigs, respectively, these MAbs localized almost exclusively to the tumor vasculature (Thorpe, *et al. unpublished*). Retroviral vectors requiring cell division for transgene expression enter target cells 'piggybacking' on a

15   mitogenic factor, VEGF, and are integrated into the genome of target cells at high efficiency.

Suitable tumor markers to target include, but are not limited to, c-erbB2/p185[HER-2] (Shepard, *et al., J. Clin. Immun. 11*:117, 1991), TAG-72 (Guadagni, *et al., Int. J. Biol. Markers 9*:53, 1994), carcinoembryonic antigen (CEA, Guadagni, *et al., supra*), the high

20   affinity folate receptor (Bottero, *et al., Cancer Res. 53*:5791, 1993), polymorphic epithelial mucin (PEM, Rettig, *et al., Current Opinion Immunology 4*:630, 1992), epidermal growth factor receptor (Rettig, *et al., supra*), a CD44 isoform (Rettig, *et al., supra*) and prostate-specific antigen (Mulders, *et al., Eur. J. Surg. Onco. 16*:37, 1990). Targeting may be achieved with the appropriate biotinylated MAbs complexed to retroviral vectors with

25   chimeric *env*-avidin receptors (U.S.S.N. 08/242,407), or a direct *env*-ligand Chimera-containing (*e.g.*, heregulin, the ligand for c-erbB2) recombinant vector could be used. A particularly preferred targeting ligand is folic acid because it is readily available and can be attached to recombinant vectors by a relatively mild chemical procedure (Lee, *et al., Cancer Res. 52*:5954, 1992). Importantly, the serum half-life of the vector may be limited

30   without loss of activity since the target subpopulation of tumor cells is one of the most readily accessible extravascular tissues in the body. Targeting to perivascular tumor cells of a procoagulant protein whose site of action is "upstream" (*i.e.*, in the lumen of the adjacent capillary) has been shown to be effective *in vivo* using a bispecific anti-tumor/anti-tissue factor antibody (Molema, *et al.*, submitted for publication) because clotting factors leak

35   into the perivascular tumor interstium through the hyperpermeavle tumor vascularity (Senger, *et al., supra*).

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Alternatively, targeting may also be achieved using hybrid envelopes (WO 92/14829) carrying other ligands for target receptors such as heregulin (bind her-2-neu) or EPO (binds the EPO receptor) (WO 93/25234). Such hybrid envelopes may need to be combined with an unmodified envelope to give the required performance. Even though the receptor ligand affinity may not be of the order that is deemed desirable for monoclonal antibody targeting, it may be sufficient to target the vector because of the multiple ligand receptor interactions with the target cells. This "velcro" effect is known to give vector-cell affinities in 10^{-10} to 10^{-15} range as measured by Scatchard blots (Yu, H., et al., Proceedings of 1st West Coast Retrovirus Meeting, Eds. H. Fan and M. Linial, 1994)

Several features of this strategy are optimal for recombinant vector gene therapy. In particular, it is important that the procoagulant protein be synthesized close to the vasculature and this will occur because the perivascular tumor cells are the most physically accessible tumor cells to circulating vector particles and are rapidly proliferating, thus being particularly susceptible to recombinant viral infection.

Since tumor cells are suseptable to integration because of their increased proliferation, gene therapy using recombinant retroviral vectors based on the C-type viruses (e.g., MLV) may be used without targeting. Because only cycling cells will integrate the vector, non-dividing normal cells will be unaffected. Other proliferating epithelial cells (e.g., in hair follicles and the lumen of the gut) are separated from extravasating vector particles by several layers of stromal tissue. Consequently, it is unlikely that the vector would penetrate deeply enough into the tissue to be able to integrate in such cells (Burrows, et al., Cancer Res. 52:5954, 1992).

The present invention further provides recombinant vectors in a variety of viral vector packaging systems in which one or more essential functions of the parent virus has been deleted so that it is deficient in some function (e.g., genome replication), but retains a packaging signal and the ability to express a heterologous inserted gene sequence (the "gene of interest"). The deleted essential function or functions are provided by packaging cells into which the vector genome can be introduced to yield producer cell lines that then make replication defective viral particles encapsidating the recombinant vector. The recombinant vector may further contain one or more nucleic acid cassettes described above. The vector genome is then introduced into target cells by an infection event ("transduction") but is incapable of further propagation. In any such situation, it is important to prevent the recombination of the various parts of the virus in a producer cell line to give replication competent virus genomes, or to eliminate cells in which this occurs. Many such vectors, packaging cells and producer cells, may be constructed for a variety of viruses, including for example, poliovirus (Evans et al., Nature 339:385, 1989, and Sabin et al., J. of Biol. Standardization 1:115, 1973) (ATCC VR-58); rhinovirus (Arnold et al.,

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J. Cell. Biochem. L401, 1990) (ATCC VR-1110); pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch *et al.*, *PNAS* 86:317, 1989; Flexner *et al.*, *Ann. N.Y. Acad. Sci.* 569:86, 1989; Flexner *et al.*, *Vaccine* 8:17, 1990; U.S. 4,603,112 and U.S. 4,769,330; WO 89/01973) (ATCC VR-111; ATCC VR-2010); SV40 (Mulligan *et al.*, *Nature* 277:108,

5   1979) (ATCC VR-305), (Madzak *et al.*, *J. Gen. Vir.* 73:1533, 1992); influenza virus (Luytjes *et al.*, *Cell* 59:1107, 1989; McMicheal *et al.*, *The New England Journal of Medicine* 309:13, 1983; and Yap *et al.*, *Nature* 273:238, 1978) (ATCC VR-797); adenovirus (Berkner *et al.*, *Biotechniques* 6:616, 1988, and Rosenfeld *et al.*, *Science* 252:431, 1991) (ATCC VR-1); parvovirus such as adeno-associated virus (Samulski *et al.*,

10 *J. Vir.* 63:3822, 1989, and Mendelson *et al.*, *Virology* 166:154, 1988) (ATCC VR-645); Human Immunodeficiency virus; herpes simplex virus (Kit *et al.*, *Adv. Exp. Med. Biol.* 215:219, 1989) (ATCC VR-977; ATCC VR-260); *Nature* 277: 108, 1979); HIV (EPO 386,882, Buchschacher *et al.*, *J. Vir.* 66:2731, 1992); measles virus (EPO 440,219) (ATCC VR-24); Sindbis virus (ATCC VR-68; ATCC VR-1248; Xiong, *et al.*, *Science* 234:1188,

15 1989) Semliki Forest virus (ATCC VR-67; ATCC VR-1247), Aura (ATCC VR-368), Bebaru virus (ATCC VR-600; ATCC VR-1240), Cabassou (ATCC VR-922), Chikungunya virus (ATCC VR-64; ATCC VR-1241), Fort Morgan (ATCC VR-924), Getah virus (ATCC VR-369; ATCC VR-1243), Kyzylagach (ATCC VR-927), Mayaro (ATCC VR-66), Mucambo virus (ATCC VR-580; ATCC VR-1244), Ndumu (ATCC VR-371), Pixuna

20 virus (ATCC VR-372; ATCC VR-1245), Tonate (ATCC VR-925), Triniti (ATCC VR-469), Una (ATCC VR-374), Whataroa (ATCC VR-926), Y-62-33 (ATCC VR-375), Middleberg virus (ATCC VR-370), Ross River virus (ATCC VR-373; ATCC VR-1246), O'Nyong virus, Eastern encephalitis virus (ATCC VR-65; ATCC VR-1242), Western encephalitis virus (ATCC VR-70; ATCC VR-1251; ATCC VR-622; ATCC VR-1252) or

25 Venezuelan equine encephalitis virus (ATCC VR923; ATCC VR-1250; ATCC VR-1249; ATCC VR-532), and coronavirus (Hamre *et al.*, *Proc. Soc. Exp. Biol. Med.* 121:190, 1966) (ATCC VR-740). In particular, such vectors, packaging cells and producer cells may be constructed from a variety of retroviruses, including avian leukosis virus (ATCC Nos. VR-535 and VR-247), BLV (VR-1315), bovine leukemia virus, MLV, mink-cell

30 focus-inducing virus (Koch *et al.*, *J. Vir.* 49:828, 1984; and Oliff *et al.*, *J. Vir.* 48:542, 1983), murine sarcoma virus (ATCC Nos. VR-844, 45010 and 45016), reticuloendotheliosis virus (ATCC Nos VR-994, VR-770 and 45011), gibbon ape leukemia virus, Mason-Pfizer leukemia virus, and rous sarcoma virus (ATCC Nos VR-772, VR-354, VR-270, VR-724 and VR-725). Particularly preferred retroviruses are murine leukemia

35 viruses 4070 A and 1504 A (Hartley, *J. Vir.* 19:19, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi (Ru *et al.*, *J. Vir.* 67:4722, 1993; and Yantchev *Neoplasma* 26:397, 1979), Gross (ATCC No. VR-590), Kristen (Albino *et al.*, *J. Exp.*

Med. 164:1710, 1986), Harvey sarcoma virus (Manly *et al.*, *J. Vir. 62*:3540, 1988; and Albino *et al.*, *J. Exp. Med. 164*:1710, 1986) and Raucher (ATCC No. VR-998) and Moloney leukemia virus (ATCC No. VR-190). Particularly preferred non-mouse retroviruses are rous sarcoma viruses, Bratislava, (Copeland *et al.*, *J. Neuropath. Exp. Neurology 34*:N1, P100, 1975), Bryan high titer (*e.g.*, ATCC Nos. VR-334, VR-657, VR-726, VR-659, and VR-728), Bryan standard (ATCC No VR-140), Carr-Zilber (Adgighitov *et al.*, *Neoplasma 27*:159, 1980, Engelbreth-Holm-Swarm (Laurent *et al.*, *Biochem Biophys Acta* 908:241, 1987), Harris, Prague (ATCC Nos. VR-772 and 45033), Schmidt-Ruppin (*e.g.*, ATCC Nos. VR-724, VR-725, and VR-354). The expression vector may be readily assembled from any virus or retrovirus utilizing standard recombinant techniques (*e.g.*, Sambrook *et. al.*, *Molecular Cloning: A Laboratory Manual*, 2d ed. Cold Spring Harbor Laboratory Press, 1989). Further description of the construction of retroviral vectors is described in U.S.S.N. 07/586,603, herein incorporated by reference.

Within a preferred embodiment of the present invention, the recombinant vector is a recombinant retroviral vector. These constructs carry or express a selected gene(s) or sequence(s) of interest. Numerous retroviral gene delivery vehicles may be utilized within the context of the present invention, including for example EP 415,731; WO 90/07936; WO 91/0285, WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 9310218; Vile and Hart, *Cancer Res. 53*:3860-3864, 1993; Vile and Hart, *Cancer Res. 53*:962-967, 1993; Ram *et al.*, *Cancer Res. 53*:83-88, 1993; Takamiya *et al.*, *J. Neurosci. Res. 33*:493-503, 1992; Baba *et al.*, *J. Neurosurg. 79*:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 345,242 and WO91/02805).

Retroviral gene delivery vehicles of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (*see* RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Briefly, viruses are often classified according to their morphology as seen under electron microscopy. Type "B" retroviruses appear to have an eccentric core, while type "C" retroviruses have a central core. Type "D" retroviruses have a morphology intermediate between type B and type C retroviruses. Representative examples of suitable retroviruses include those discussed in RNA Tumor Viruses, *supra*, as well as a variety of xenotropic retroviruses (*e.g.*, NZB-X1, NZB-X2 and NZB$_{9-1}$ (*see* O'Neill *et al.*, *J. Vir. 53*:100-106, 1985)) and polytropic retroviruses (*e.g.*, MCF and MCF-MLV (*see* Kelly *et al.*, *J. Vir. 45*(1):291-298, 1983)). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection (ATCC, Rockville, MD), or isolated from known sources using commonly available techniques.

Particularly preferred retroviruses for the preparation or construction of retroviral gene delivery vehicles of the present invention include retroviruses selected from the group consisting of Avian Leukosis Virus, Bovine Leukemia Virus, Murine Leukemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis virus and

5   Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe, *J. Virol.* *19*:19-25, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten, Harvey Sarcoma Virus and Rauscher (ATCC No. VR-998), and Moloney Murine Leukemia Virus (ATCC No. VR-190). Particularly preferred Rous Sarcoma Viruses include Bratislava,

10   Bryan high titer (*e.g.*, ATCC Nos. VR-334, VR-657, VR-726, VR-659, and VR-728), Bryan standard, Carr-Zilber, Engelbreth-Holm, Harris, Prague (*e.g.*, ATCC Nos. VR-772, and 45033), and Schmidt-Ruppin (*e.g.* ATCC Nos. VR-724, VR-725, VR-354).

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral gene delivery vehicles given the disclosure provided herein, and

15   standard recombinant techniques (*e.g.*, Sambrook *et al*, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS 82*:488, 1985). In addition, within certain embodiments of the invention, portions of the retroviral gene delivery vehicles may be derived from different retroviruses. For example, within one embodiment of the invention, recombinant retroviral vector LTRs may be derived from a

20   Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus.

Within one aspect of the present invention, retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous

25   sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein the vector construct lacks *gag/pol* or *env* coding sequences. Briefly, Long Terminal Repeats ("LTRs") are subdivided into three elements, designated U5, R and U3. These elements contain a variety of signals which are responsible for the biological activity of a retrovirus, including for example, promoter and enhancer elements which are located within U3.

30   LTRs may be readily identified in the provirus (integrated DNA form) due to their precise duplication at either end of the genome. As utilized herein, a 5' LTR should be understood to include a 5' promoter element and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector. The 3' LTR should be understood to include a polyadenylation signal, and sufficient LTR sequence to allow

35   reverse transcription and integration of the DNA form of the vector.

The tRNA binding site and origin of second strand DNA synthesis are also important for a retrovirus to be biologically active, and may be readily identified by one of

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skill in the art. For example, retroviral tRNA binds to a tRNA binding site by Watson-Crick base pairing, and is carried with the retrovirus genome into a viral particle. The tRNA is then utilized as a primer for DNA synthesis by reverse transcriptase. The tRNA binding site may be readily identified based upon its location just downstream from the 5'

5 LTR. Similarly, the origin of second strand DNA synthesis is, as its name implies, important for the second strand DNA synthesis of a retrovirus. This region, which is also referred to as the poly-purine tract, is located just upstream of the 3' LTR.

In addition to a 5' and 3' LTR, tRNA binding site, and origin of second strand DNA synthesis, certain preferred recombinant retroviral vector constructs which are provided

10 herein also comprise a packaging signal, as well as one or more heterologous sequences, each of which is discussed in more detail below.

Within one aspect of the invention, recombinant retroviral vector constructs are provided which lack both *gag/pol* and *env* coding sequences. As utilized herein, the phrase "lacks *gag/pol* or *env* coding sequences" should be understood to mean that the

15 recombinant retroviral vector does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably at least 8 consecutive nucleotides which are found in *gag/pol* or *env* genes, and in particular, within *gag/pol* or *env* expression cassettes that are used to construct packaging cell lines for the recombinant retroviral vector constructs are set forth in more detail below and in Example 1.

20 As an illustration, within one embodiment of the invention construction of recombinant retroviral vector constructs which lack *gag/pol* or *env* sequences may be accomplished by preparing vector constructs which lack an extended packaging signal. As utilized herein, the phrase "extended packaging signal" refers to a sequence of nucleotides beyond the minimum core sequence which is required for packaging, that allows increased

25 viral titer due to enhanced packaging. As an example, for the Murine Leukemia Virus MoMLV, the minimum core packaging signal is encoded by the sequence beginning from the end of the 5' LTR (approximately nucleotide 144), up through the *Pst* I site (nucleotide 567). The extended packaging signal of MoMLV includes the sequence beyond nucleotide 567 up through the start of the *gag/pol* gene (nucleotide 621), and beyond nucleotide 1560.

30 Thus, within this embodiment recombinant retroviral vector constructs which lack extended packaging signal may be constructed from the MoMLV by deleting or truncating the packaging signal prior to nucleotide 567.

Within other embodiments of the invention, recombinant retroviral vector constructs are provided wherein the packaging signal that extends into, or overlaps with,

35 retroviral *gag/pol* sequence is deleted or truncated. For example, in the representative case of MoMLV, the packaging signal is deleted or truncated prior to the start of the *gag/pol*

gene (nucleotide 621). Within preferred embodiments of the invention, the packaging signal is terminated at nucleotide 570, 575, 580, 585, 590, 595, 600, 610, 615 or 617.

Within other aspects of the invention, recombinant retroviral vector constructs are provided which include a packaging signal that extends beyond the start of the *gag/pol* gene (*e.g.*, for MoMLV, beyond nucleotide 621). When such recombinant retroviral vector constructs are utilized, it is preferable to utilize packaging cell lines for the production of recombinant viral particles wherein the 5' terminal end of the *gag/pol* gene in a *gag/pol* expression cassette has been modified to contain codons which are degenerate for *gag*.

Within other aspects of the present invention, recombinant retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the recombinant retroviral vector construct does not contain a retroviral nucleic acid sequence upstream of the 5' LTR. As utilized within the context of the present invention, the phrase "does not contain a retroviral nucleic acid sequence upstream of the 5' LTR" should be understood to mean that the recombinant retroviral vector does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably at least 8 consecutive nucleotides which are found in a retrovirus, and more specifically, in a retrovirus which is homologous to the recombinant retroviral vector construct. Within a preferred embodiment, the recombinant retroviral vector constructs do not contain a *env* coding sequence upstream of the 5' LTR. A particularly preferred embodiment of such recombinant retroviral vector constructs is set forth in more detail below in Example 1.

Within a further aspect of the present invention, recombinant retroviral vector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the recombinant retroviral vector construct does not contain a retroviral packaging signal sequence downstream of the 3' LTR. As utilized herein, the term "packaging signal sequence" should be understood to mean a sequence sufficient to allow packaging of the RNA genome. A representative example of such a recombinant retroviral vector construct is set forth in more detail below in Example 1.

Packaging cell lines suitable for use with the above described recombinant retroviral vector constructs may be readily prepared (*see* U.S. Serial No. 08/240,030, filed May 9, 1994; *see also* U.S. Serial No. 07/800,921, filed November 27, 1991), and utilized to create producer cell lines (also termed vector cell lines or "VCLs") for the production of recombinant vector particles.

Within another preferred aspect of the invention, the recombinant viral vector is a recombinant alphavirus viral vector based on Sindbis virus, which may function as gene delivery vehicles. Briefly, the Sindbis virus is the prototype member of the alphavirus

genus of the togavirus family. The unsegmented genomic RNA (49S RNA) of Sindbis virus is approximately 11,703 nucleotides in length, contains a 5' cap and a 3' poly-adenylated tail, and displays positive polarity. Infectious enveloped Sindbis virus is produced by assembly of the viral nucleocapsid proteins onto the viral genomic RNA in the

5 cytoplasm and budding through the cell membrane embedded with viral encoded glycoproteins. Entry of virus into cells is by endocytosis through clatharin coated pits, fusion of the viral membrane with the endosome, release of the nucleocapsid, and uncoating of the viral genome. During viral replication the genomic 49S RNA serves as template for synthesis of the complementary negative strand. This negative strand in turn serves as

10 template for genomic RNA and an internally initiated 26S subgenomic RNA. The Sindbis viral nonstructural proteins are translated from the genomic RNA while structural proteins are translated from the subgenomic 26S RNA. All viral genes are expressed as a polyprotein and processed into individual proteins by post translational proteolytic cleavage. The packaging sequence resides within the nonstructural coding region,

15 therefore only the genomic 49S RNA is packaged into virions.

        Several different alphavirus vector systems may be constructed and utilized within the present invention. Representative examples of such systems include those described within U.S. Patent Nos. 5,091,309 and 5,217,879.

        Particularly preferred alphavirus vectors for use within the present invention include

20 those which are described within WO 95/07994 and U.S. Serial No. 08/405,627. Briefly, within one embodiment, Sindbis vector constructs are provided comprising a 5' sequence which is capable of initiating transcription of a Sindbis virus, a nucleotide sequence encoding Sindbis non-structural proteins, a viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, and a Sindbis RNA

25 polymerase recognition sequence. Within other embodiments, the viral junction region has been modified such that viral transcription of the subgenomic fragment is reduced, increased, or maintained. Within another embodiment, Sindbis vector constructs are provided comprising a 5' sequence which is capable of initiating transcription of a Sindbis virus, a nucleotide sequence encoding Sindbis non-structural proteins, a first viral junction

30 region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, a second viral junction region which has been modified such that viral transcription of the subgenomic fragment is reduced, and a Sindbis RNA polymerase recognition sequence. Within yet another embodiment, Sindbis cDNA vector constructs are provided comprising the above-described vector constructs, in addition to a 5' promoter

35 which is capable of initiating the synthesis of viral RNA from cDNA, and a 3' sequence which controls transcription termination.

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In still further embodiments, the vector constructs described above contain no Sindbis structural proteins in the vector constructs the selected heterologous sequence may be located downstream from the viral junction region; in the vector constructs described above having a second viral junction, the selected heterologous sequence may be located
5   downstream from the second viral junction region, where the heterologous sequence is located downstream, the vector construct may comprise a polylinker located between the viral junction region and said heterologous sequence, and preferably the polylinker does not contain a wild-type Sindbis virus restriction endonuclease recognition sequence.

The above described Sindbis vector constructs, as well as numerous similar vector
10 constructs, may be readily prepared essentially as described in U.S. Serial No. 08/405,627, which is incorporated herein by reference in its entirety.

In addition to the above viral-based vectors, numerous non-viral gene delivery vehicles may likewise be utilized within the context of the present invention. Representative examples of such gene delivery vehicles include direct delivery of nucleic
15 acid expression vectors, naked DNA alone (WO 90/11092), polycationic lipids and liposomes encapsulating nucleic acid (single or double stranded DNA or RNA) expression vectors, polycation condensed DNA linked or unlinked to killed adenovirus (Curiel et al., Hum. Gene Ther. 3:147-154, 1992), DNA linked to a ligand with or without one of the high affinity pairs described above (Wu et al., J. of Biol. Chem 264:16985-16987, 1989),
20 and certain eukaryotic cells (e.g., producer cells - see U.S. Serial No. 08/240/030, filed May 9, 1994, and U.S. Serial No. 07/800,921). All such DNA vectors can be delivered as a gene delivery vehicle where an initial RNA pol II transcription encodes a viral bacteriophage or other source RNA replicase and in the same or a separate transcript, an RNA message that can be amplified in the cytoplasm by the RNA replicase, encoding the
25 desired gene to be explored for therapy (U.S.S.N. 08/404,796).

When a gene delivery vehicle according to the invention is targeted to a tumor, a variety of strategies can be utilized. In one embodiment, to target the gene delivery vehicle to the tumor, a targeting element is utilized to specifically direct a gene delivery vehicle to a selected cell type. Generally, targeting elements are proteins or peptides, although other
30 non-proteinaceous molecules may also function as targeting elements. A wide variety of targeting elements are known in the art. For example, within one embodiment of the invention, antibodies may be utilized in order to target a selected cell type (see generally, Wilchek and Bayer, Anal. Biochem 171:1-32, 1988). Representative examples include anti-CD34 antibodies (e.g., 12.8 (Andrews et al., Blood 67:842, 1986), and My10 (Civin et
35 al., J. Immunol. 133:157, 1984; commercially available from Becton Dickinson under the designation HPCA-2)) which may be utilized to target the anti-CD34 antigen on stem cells, the anti-CD4 antibody which may be utilized to target CD4+ T-cells, anti-CD8 antibodies

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to target CD8+ cells the HER2/neu monoclonal antibody 4D5 (Sarup, *et al.*, *Growth Regul. 1*:72, 1991) to target ovarian and breast cells, the c-erbB-2 monoclonal antibody GDF-OA-p185-1 (Alper, *et al.*, *Cell Growth Differ. 1*:591, 1990) to target breast cells, the TAG72 monoclonal Ab: CC49 and B72.3 (King, *et al.*, *J. Biochem. 281*:317, 1992) to 5 target colon and breast cells, and the carcinoembryonic antigen monoclonal antibody ZCE025 (Nap, *et al.*, *Canc. Res. 52*:2329, 1992) to target colon carcinoma cells.

As an alternative to the use of targeting elements, a high affinity binding pair can be employed. A wide variety of high affinity binding pairs are known in the art. Representative examples of suitable affinity binding pairs include biotin/avidin with an 10 affinity (K_D) of 10^{-15} M (Richards, *Meth. Enz. 184*:3-5, 1990; Green, *Adv. in Protein Chem. 29*:85, 1985); cytostatin/papain with an affinity of 10^{-14}M (Bjork and Ylinenjarvi, *Biochemistry 29*:1770-1776, 1990); val-phosponate/ carboxypeptidase A with an affinity of 10^{-14} M (Kaplan and Bartlett, *Biochemistry 30*:8165-8170, 1991); 4CABP-RuBisCo with an affinity of 10^{-13} M, (Schloss, *J. Biol. Chem. 263*:4145-4150, 1988); and tobacco 15 hornworm diuretic hormone/tobacco hornworm diuretic hormone receptor, with an affinity of 10^{-11}M (Reagan *et al.*, *Arch. Insect Biochem. Physiol. 23*:135-145, 1993).

A wide variety of other high affinity binding pairs may also be developed, for example, by preparing and selecting antibodies which recognize a selected antigen, and by further screening of such antibodies in order to select those with a high affinity (*see* 20 generally, U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993; *see* also *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Alternatively, antibodies or antibody fragments may also be produced and selected utilizing 25 recombinant techniques (*see* William D. Huse *et al.*, *Science 246*:1275-1281, December 1989; *see also* L. Sastry *et al.*, *Proc. Natl. Acad. Sci. USA 86*:5728-5732, 1989; *see also* Michelle Alting-Mees *et al.*, *Strategies in Molecular Biology 3*:1-9, 1990; these references describe a commercial system available from Stratacyte, La Jolla, California, which enables the production of antibodies through recombinant techniques).

30 As will be evident to one of ordinary skill in the art given the disclosure provided herein, either member (or molecule) of the affinity binding pair may be coupled to the gene delivery vehicle (or conversely, the targeting element). Nevertheless, within preferred embodiments of the invention, the larger of the two affinity binding pairs (*e.g.*, avidin of the avidin/biotin pair) is coupled to gene delivery vehicle.

35 As noted above, certain embodiments of the present invention provide gene delivery vehicles which have been coupled to a member of a high affinity binding pair (also referred to as the "coupled gene delivery vehicle"), as well as targeting elements which have coupled

to a member of a high affinity binding pair (also referred to as the "coupled targeting element"). As utilized within the context of the present invention, the term "coupled" may refer to either noncovalent or covalent interactions, although generally covalent bonds are preferred. Numerous methods may be utilized in order to couple one member of a high
5   affinity binding pair to either a gene delivery vehicle or a targeting element, including for example use of crosslinking agents such as N-succinimidyl-3-(2-pyridyl dithio) propionate ("SPDP"; Carlson *et al., J. Biochem. 173*:723, 1978); Sulfosuccinimidyl 4-N-maleimidomethyl) cyclohexane-1-carboxylate ("SulfoSMCC"); 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide ("EDC"); Bis-diazobenzidine ("BDB"); and Periodic
10 acid / Schiff's base.

        Within particularly preferred embodiments of the invention, a member of the high affinity binding pair is either expressed on, or included as an integral part of, the exterior (*e.g.*, envelope) of the gene delivery vehicle. For example, within one embodiment of the invention a member of the affinity binding pair is co-expressed along with the envelope
15 protein of a viral gene delivery vehicle, as a hybrid protein.

        As those in the art will appreciate, other targeting technologies are also known in the art and may be employed to target a gene delivery vehicle according to the invention to a particular tumor or cell type.

        Any of the gene delivery vehicles described above may include, contain (and/or
20 express) one or more heterologous sequences. A wide variety of heterologous sequences may be utilized within the context of the present invention, including for example, nucleotide sequences which encode polypeptides capable of causing blood clot formation, inhibiting fibrinolysis, inhibiting tumor vascularization, activating a compound with little or no cytotoxicity (*i.e.*, a "prodrug") into a toxic product, and binding or metabolizing
25 nutrients in the perivascular interstitium.

        DNA molecules encoding such heterologous genes may be obtained from a variety of sources, such as cloning from plasmids deposited at recognized depositories of biological materials. Alternatively, known cDNA sequences encoding cytotoxic genes or other heterologous sequences may be obtained from cells which express or contain such
30 sequences. Briefly, within one embodiment of the invention, mRNA from a cell expressing the gene of interest is reverse transcribed with reverse transcriptase using oligo dT or random primers. The single-stranded cDNA may then be amplified by PCR (*see* U.S. 4,683,202, U.S. 4,683,195 and U.S. 4,800,159. *See also* PCR Technology: *Principles and Applications for DNA Amplification*, Erlich, Stockton Press, 1989, all of
35 which are herein incorporated by reference) utilizing oligonucleotide primers complementary to sequences upstream or downstream of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq

polymerase, sequence specific DNA primers, and nucleotide bases dATP, dCTP, dGTP and dTTP. After annealing and elongation, double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in an exponential amplification of the desired DNA.

5   Sequences which encode the above-described genes of interest may also be partially or completely chemically synthesized, for example, on an Applied Biosystems Inc. automated DNA synthesizer (*e.g.*, ABI, DNA synthesizer model 392 (Foster City, CA)). Such genes may comprise a naturally occurring nucleotide sequence, an "optimized" nucleotide sequence based on codon preference, or combination(s) of the two.

10   In another embodiment of the present invention, recombinant vectors may direct the expression of more than one heterologous sequence. Such multiple sequences may be controlled either by a single promoter (whether it is present in the vector or in the genome into which the vector is integrated), or preferably, by one or more additional promoters and may also include internal ribosome binding sites ("IRBS") in the event polycistronic 15 messages are employed. Such sequence are small, typically about 300 bp, and may readily be incorporated into a vector in order to express multiple genes from a multicistronic message whose cistrons begin with this sequence (Jacejak and Sarnow, *Nature* 353:90, 1991).

As noted above, several anti-tumor agents may be administered either concurrently 20 or sequentially in order to inhibit the growth of a selected tumor in accordance with the methods of the present invention. For example, an anti-tumor agent such as γ-IFN may be co-administered or sequentially administered to an animal along with other anti-tumor agents such as IL-2, or IL-12, in order to inhibit or destroy the tumor in conjunction with recombinant vectors described herein. Such therapeutic compositions may be administered 25 directly using a single vector which directs the expression of two or more anti-tumor agents or the anti-tumor agents may be expressed by independent vectors. Alternatively, an anti-tumor agent according to the invention, is expressed by a recombinant vector administered to the animal, while other tumor agents (*e.g.*, IL-2, γ-IFN) are administered (*e.g.*, intravenously) as a pharmaceutical composition.

30   In another embodiment of the invention, a recombinant vector carried by the gene delivery vehicle may also be administered to a patient that expresses two anti-tumor agents. In such a vector, the first anti-tumor agent may be expressed from an LTR present in the recombinant vector and the other agent may utilize an additional transcriptional promoter located between the LTRs. Alternatively, the second anti-tumor agent may be expressed as 35 a polycistronic mRNA, which may incorporate one or more internal ribosome binding sites. Briefly, with respect to IRBS, the upstream untranslated region of the immunoglobulin heavy chain binding protein has been shown to support the internal engagement of a

bicistronic message (see Jacejak and Sarnow, *Nature 353*:90, 1991). This sequence is small (300 bp), and may readily be incorporated into a retroviral vector in order to express multiple genes from a multi-cistronic message whose cistrons begin with this sequence. A representative vector construct utilizing IRBS is set forth in more detail below in Examples 5(A)(i), 5(B)(i), 5(C)(i), and 5(D)(i).

In another embodiment of the invention, pharmaceutical compositions are provided comprising a gene delivery vehicle in combination with a pharmaceutically acceptable carrier or diluent. Such pharmaceutical compositions may be prepared in liquid or solid form (*e.g.*, lyophilized). The solid form is suspended in a solution prior to parenteral administration. A therapeutically effective amount the gene delivery vehicle will be administered via I.V., intra-arterially (I.A.), or intralymphatically (I.L.). A therapeutically effective amount of the gene delivery vehicle is an amount sufficient to halt tumor grwoth and preferably kill the tumor cells. Typical dosages are 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, or 10^{11} cfu for vectors that carry a selectable marker and equivalent titer by PCR plaque formation or equivalent methods for those that do not. Nucleic acid vectors are delivered at doses of 0.1, 1.0, 10, 100, 1,000, or 10,000 µg.

Pharmaceutically acceptable carriers or diluents are non-toxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic saline solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, lactose, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin. A particularly preferred composition comprises a vector or recombinant virus in 40 mg/ml mannitol or lactose, 5 mg/ml HSA, 25 mM Tris, pH 7.2, 1 mg/ml arginine and 25 to 75 mM NaCl. This composition is stable at -70°C for at least six months.

A variety of tumors may be selected for treatment in accordance with the methods described herein. In general, solid tumors are preferred, although leukemias and lymphomas may also be treated if they have developed a solid mass, or if suitable tumor associated markers exist such that the tumor cells can be physically separated from nonpathogenic normal cells. For example, acute lymphocytic leukemia cells may be sorted from other lymphocytes with the leukemia specific marker 'CALLA'. Representative examples of suitable tumors include melanomas, colorectal carcinomas, lung carcinomas (including large cell, small cell, squamous and adeno-carcinomas), renal cell carcinomas and breast adeno-carcinomas.

The following examples are offered by way of illustration, and provide preferred embodiments of the invention but are not meant to limit the scope thereof. Standard methods for many of the procedures mentioned or described in the following examples, or

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suitable alternative procedures, are provided in widely recognized manuals of molecular biology such as, for example, Sambrook *et al.*, "Molecular Cloning," Second Edition, Cold Spring Harbor Laboratory Press (1997) and in Asubel *et al.*, (Eds.), "Current Protocols in Molecular Biology," Greene Associates/Wiley Interscience, New York (1990).

5

EXAMPLES

Example 1
Construction of Recombinant Vectors

10

A.   Preparation of Retroviral Backbones

i.   Preparation of Retroviral Backbones KT-1 and KT-3B

15

The Moloney murine leukemia virus (MoMLV) 5' long terminal repeat (LTR) EcoR I-EcoR I fragment, including *gag* sequences, from the N2 (Armentano, *et al., J. Vir.* *61*:1647-1650, 1987; Eglitias, *et al., Science 230*:1395-1398, 1985) vector is ligated into the plasmid SK$^+$ (Stratagene, San Diego, CA). The resulting construct is designated
20   N2R5. The N2R5 construct is mutated by site-directed *in vitro* mutagenesis to change the ATG start codon to ATT, preventing *gag* expression. This mutagenized fragment is 200 bp in length and flanked by Pst I restriction sites. The Pst I-Pst I mutated fragment is purified from the SK$^+$ plasmid and inserted into the Pst I site of N2 MoMLV 5' LTR in plasmid pUC31 to replace the non-mutated 200 bp fragment. The plasmid pUC31 is
25   derived from pUC19 (Stratagene, San Diego, CA) in which additional restriction sites Xho I, Bgl II, BssH II and Nco I are inserted between the EcoR I and Sac I sites of the polylinker. This construct is designated pUC31/N2R5gM.

A 1.0 Kb MoMLV 3' LTR EcoR I-EcoR I fragment from N2 is cloned into plasmid SK$^+$ resulting in a construct designated N2R3$^-$. A 1.0 kilobase (Kb) Cla I-Hind III
30   fragment is purified from this construct.

The Cla I-Cla I dominant selectable marker gene fragment from pAFVXM retroviral vector, comprising a SV40 early promoter driving expression of the neomycin (*neo*) phosphotransferase gene, is cloned into the SK$^+$ plasmid. This construct is designated SV$^+$ SV$_2$-*neo* . A 1.3 Kb Cla I-BstB I gene fragment is purified from the SK$^+$
35   SV$_2$-*neo* plasmid.

KT-3B or KT-1 vectors are constructed by a three part ligation in which the Xho I-Cla I fragment containing the gene of interest and the 1.0 Kb MoMLV 3' LTR Cla I-Hind

III fragment are inserted into the Xho I-Hind III site of pUC31/N2R5gM plasmid. This gives a vector designated as having the KT-1 backbone. The 1.3 Kb Cla I-BstB I *neo* gene fragment from the pAFVXM retroviral vector is then inserted into the Cla I site of this plasmid in the sense orientation to yield a vector designated as having the KT-3B

5   backbone.

    ii.   <u>Preparation of Retroviral Backbone KT-3BC</u>

An alternative selectable marker, phleomycin resistance (Mulsant *et al., Som. Cell*

10   *and Mol. Gen. 14*:243, 1988, available from Cayla, Cédex, FR) may be used to make the retroviral backbone KT-3BC, for use in transforming genes to cells that are already neomycin resistant. The plasmid pUT507 (Mulsant *et al., Som. Cell and Mol. Gen. 14*:243, 1988) is digested with Nde I and the ends blunted with Klenow fragment. The sample is then digested with Hpa I and Cla I linkers are ligated to the mix of fragment. The

15   sample is then digested with Cla I to remove excess Cla I linkers. The 1.2 Kb Cla I fragment carrying the rous sarcoma virus (RSV) LTR and the phleomycin resistance gene are isolated by agarose gel electrophoresis followed by purification using GeneClean II™ (Bio101, San Diego, CA). This 1.2 Kb fragment is inserted into KT-1 instead of the 1.3 Kb Cla I-BstB I neomycin resistance fragment to give the backbone KT-3BC.

20
    iii.   <u>Preparation of Retroviral Backbones Containing a Polylinker.</u>

A polylinker sequence is inserted into each of the KT vector backbones (KT-1, KT-3B, KT-3BC) to facilitate the insertion of heterologous sequences. The polylinker is

25   constructed using two complementary oligonucleotides that form a duplex with Xho I and Cla I compatible sticky ends when hybridized.

KTLinkF:

30   (Sequence ID No.: 1)

       5'-TCGAGCGCTATGCATGTTTAAACGCGTGCGGCCGCACGTGAT-3'

KTLinkR:

35
(Sequence ID No.: 2)

       5'-CGATCACGTGCGGCCGCACGCGTTTAAACATGCATAGCGC-3'

The oligonucleotides are phosphorylated with T4 polynuclotide kinase, heated to 90_C, and slow cooled to allow hybridization to occur. The hybrid is then ligated to the appropriate KT-1, KT-3B, or KT-3BC vector backbone fragment obtained after digestion with Xho I and Cla I, followed by treatment with alkaline phosphatase and agarose gel purification. The resulting constructs contain Xho I, Eco47 III, Nsi I, Pme I, Mlu I, Not I, Pml I, and Cla I, as unique sites for the insertion of heterologous sequences. These constructs are designated pKT-1L, pKT-3BL, and pKT-3BCL, respectively.

B.   Construction of a Retroviral Vector Containing HSVTK

i.   Construction of Plasmids Containing Vector LTR Sequences

The following retroviral vector is based on the N2 vector. Briefly, 5' and 3' Eco RI LTR fragment (2.8 and 1.0 Kb, respectively) (Armentano, *J. Vir. 61*:1647, 1987; Eglitis, *Science 230*:1395, 1985) are initially subcloned into the Eco RI site of plasmids SK+ and pUC31. pUC31 is a modification of pUC19 carrying additional restriction sites (Xho I, Bgl II, BssH II, and Nco I) between the Eco RI and Sac I sites of the polylinker. Plasmid N2R3+/- is thereby created from ligation of the SK+ plasmid with the 1.0 Kb 3' LTR fragment. The plasmids p31N2R5+/- and p31N2R3+/- are constructed from the ligation of pUC31 with the 2.8 Kb 5' LTR and packaging signal (Ψ) or the 1.0 Kb 3' LTR fragment, respectively. In each case N2 refers to the vector source, R refers to the fact that the fragment is an Eco RI fragment, 5 and 3 refer to 5' or 3' LTRs, and + or - refers to the orientation of the insert (see Figures 2-7 for examples of LTR subclones).

In one case, a 1.2 Kb Cla I/Eco RI 5' LTR and Ψ fragment from N2 is subcloned into the same sites of an SK+ vector. This vector is designated pN2CR5. In another case, the 5' LTR containing a 6 base pair (bp) deletion of the splice donor sequence (Yee *et al.*, Cold Spring Harbor, Quantitative Biology, 51:1021, 1986) is subcloned as a 1.8 Kb Eco RI fragment into pUC31. This vector is designated p31N25Δ[+] (Figure 7).

ii.   Construction of Plasmids Containing HSVTK

The coding region and transcriptional termination signals of HSV-1 thymidine kinase gene (HSVTK) are isolated as a 1.8 Kb Bgl II/Pvu II fragment from plasmid 322TK (3.5 Kb Bam HI fragment of HSV-1 (McKnight *et al., Nuc. Acids 8*:5949, 1980) cloned into Bam HI of pBR322 (ATCC No. 31344)) and cloned into Bgl II/Sma I-digested pUC31. This construct is designated pUCTK. For constructs which require deletion of the terminator signals, pUCTK is digested with Sma I and Bam HI and the 0.3 Kb fragment

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containing the (A)$_n$ signal is removed. The remaining coding sequences and Bam HI overhang are reconstituted with a double-stranded oligonucleotide made from the following oligomers:

5   (Sequence ID No.: 3)

5' GAG AGA TGG GGG AGG CTA ACT GAG 3'

(Sequence ID No.: 4)

10

5' GAT CCT CAG TTA GCC TCC CCC ATC TCT C 3'

The resulting construct is designated pTKΔ A (Figure 8).

For diagnostic purposes, the oligonucleotides are designed to destroy the Sma I site
15   while maintaining the Ava I site without changing the translated protein.

The plasmid pPrTKΔA (Figure 9), which contains the HSVTK promoter and coding sequence (lacking an (A)$_n$ signal), is constructed as follows.

1.   pTKΔ A is linearized with Bgl II, treated with alkaline phosphatase, and gel purified.

20   2.   A 0.8 Kg fragment containing the HSVTK transcriptional promoter is isolated as a Bam HI/Bgl II fragment from p322TK.

3.   Products from (3) and (4) are ligated, transformed into bacteria, and positive clones are screened for the proper orientation of the promoter region. A resultant clone is designated pPrTKΔA (Figure 9).

25

iii.   Construction of Retroviral Provectors Expressing HSVTK from a Constitutive Promoter

The retroviral provectors pTK-1 and pTK-3 are constructed essentially as described
30   below.

1.   The 5 Kb Xho I/Hind III 5' LTR and plasmid sequences are isolated from p31N2R5(+) (Figure 2).

2.   HSVTK coding sequences lacking transcriptional termination sequences are isolated as a 1.2 Kb Xho I/Bam HI fragment from pTKΔA (Figure 3).

35   3.   3' LTR sequences are isolated as a 1.0 Kb Bam HI/Hind III fragment from pN2R3(-) (Figure 3).

4.   The fragments from steps 1-3 are mixed, ligated, transformed into bacteria, and individual clones identified by restriction enzyme analysis. The construct is designated pTK-1 (Figure 10).

5.   pTK-3 is constructed by linearizing pTK-1 with Bam HI, filling in the 5' overhang and blunt-end ligating a 5'-filled Cla I/Cla I fragment containing the bacterial lac UV5 promoter, SV40 early promoter, plus Tn5 neo^r gene obtained from pAFVXM retroviral vector (Krieger *et al.*, *Cell* *39*:483, 1984; St. Louis *et al.*, *PNAS* *85*:3150, 1988). Kanamycin-resistant clones are isolated and individual clones are screened for the proper orientation by restriction enzyme analysis (*see* Figure 10).

These constructs are used to generate infectious recombinant vector particles in conjunction with a packaging cell line such as DA described below.

C.   Generation of Recombinant Sindbis Vectors

i.   Cloning of a Sindbis Genomic Length cDNA

The nature of viruses having an RNA genome with positive polarity is such that when introduced into a eukaryotic cell which serves as a permissive host, the purified genomic nucleic acid serves as a functional messenger RNA (mRNA) molecule. Thus, this genomic RNA, purified from the virus, can initiate the same infection cycle which is characteristic of infection by the wild type virus from which the RNA was purified.

For example, Sindbis virus strain AR-339 (ATCC #VR-1248, Taylor *et al.*, *Am. J. Trop. Med. Hyg.* *4*:844 1955; isolated from the mosquito *Culexus univittatus*) may be propagated in baby hamster kidney-21 (BHK-21, ATCC # CCL10) cells, infected at low multiplicity (0.1 pfu/cell). Alternatively, Sindbis virus strain HR (Lee Biomolecular, San Diego, CA) may also be used and propagated by the same methods. Sindbis virions are precipitated from a clarified lysate at 48 hours post-infection with 10% (w/v) of polyethylene glycol-8000 (PEG) at 0_C, as described in U.S.S.N. 08/198,450. Sindbis virions contained in the PEG pellet may be lysed with 2% SDS, and the poly-adenylated mRNA isolated by chromatography utilizing commercially available oligo dT columns (Invitrogen, San Diego, CA).

Two rounds of first strand cDNA synthesis are performed on the polyA selected mRNA, using an oligonucleotide primer with the sequence shown below:

- 40 -

(Sequence ID No.: 5)

5'-TATATTCTAGA(dT)$_{25}$-GAAATG-3'

5 Briefly, this primer contains at its 5' end a five nucleotide 'buffer sequence' for efficient
   restriction endonuclease digestion, followed by the Xba I recognition sequence, 25
   consecutive dT nucleotides and six nucleotides which are precisely complementary to the
   extreme Sindbis 3' end. Thus, selection for first round cDNA synthesis occurs at two
   levels: (1) polyadenylated molecules, a prerequisite for functional mRNA, and (2) selective
10 priming from Sindbis mRNA molecules, in a pool containing multiple mRNA species.
   Further, the reverse transcription is performed in the presence of 10 mM MeHgOH to
   mitigate the frequency of artificial stops during reverse transcription.
       Primary genomic length Sindbis cDNA is then amplified by PCR in six distinct
   segments using six pairs of overlapping primers. Briefly, in addition to viral complementary
15 sequences, the Sindbis 5' end forward primer is constructed to contain a 19 nucleotide (nt)
   sequence corresponding to the bacterial SP6 RNA polymerase promoter and the Apa I
   restriction endonuclease recognition sequence linked to its 5' end. The bacterial SP6 RNA
   polymerase is poised such that transcription *in vitro* results in the inclusion of only a single
   non-viral G ribonucleotide linked to the A ribonucleotide, which corresponds to the
20 authentic Sindbis 5' end. Inclusion of the Apa I recognition sequence facilitates insertion of
   the PCR amplicon into the plasmid vector pKS II$^+$ (Stratagene, San Diego, CA) polylinker
   sequence. A five nt 'buffer sequence" is also inserted prior to the Apa I recognition
   sequence in order to permit efficient digestion. The sequence of the SP6-5' Sindbis forward
   primer and all of the primer pairs necessary to amplify the entire Sindbis genome are shown
25 below. The reference sequence (GenBank accession no. SINCG) is from Strauss *et al.*,
   Virology 133:92-110, 1984.

Primer	Location	Seq. ID No.	Sequence (5' to 3')	Recognition Sequence (5' to 3')
SP6-1A 1B	Apal/SP6/+SIN nt.1 to18 3,182-3,160	6 7	TATATGGGCCCGATTTAGGTGACA CTATAGATTGACGGCGTAGTACAC CTGGCAACCGGTAAGTACGATAC	Apa I Age I
2A 2B	3,144-3,164 5,905-5,885	8 9	ATACTAGCCACGGCCGGTATC TCCTCTTTCGACGTGTCGAGC	Age I Eco RI
3A 7349R	5,844-5,864 7,349-7,328	10 11	ACCTTGGAGCGCAATGTCCTG CCTTTTCAGGGGATCCGCCAC	Eco RI Bam HI
7328F 3B	7,328-7,349 9,385-9,366	12 13	GTGGCGGATCCCCTGAAAAGG TGGGCCGTGTGGTCGTCATG	Bam HI Bcl I
4A 10394R	9,336-9,356 10,394-10,372	14 15	TGGGTCTTCAACTCACCGGAC CAATTCGACGTACGCCTCACTC	Bcl I Bsi WI
10373F 4B	10,373-10,394 Xbal/dT25	16	GAGTGAGGCGTACGTCGAATTG	Bsi WI
	11,703-11,698	5	TATATTCTAGA(dT)25-GAAATG	Xba I

PCR amplification of Sindbis cDNA with the six primer sets shown above is performed in separate reactions, using the Thermalase thermostable DNA polymerase (Amresco Inc., Solon, OH) and the buffer containing 1.5 mM $MgCl_2$, provided by the supplier. Additionally, the reactions, containing 5% DMSO and Hot Start Wax beads (Perkin-Elmer, Los Angeles, CA), are performed using the following PCR amplification protocol shown below:

Temperature (°C)	Time (Min.)	No. Cycles
94	2.0	1
94	0.5	
55	0.5	35
72	3.5	
72	10.0	1

Following amplification, the six reaction products are inserted first into the pCR II vector (Invitrogen, San Diego, CA). Then using the appropriate enzymes shown above, the fragments are inserted stepwise into the pKS II$^+$ vector between the Apa I and Xba I sites. This clone is designated as pVGSP6GEN.

The Sindbis genomic cDNA clone pVGSP6GEN is linearized by digestion with Xba I, which cuts pVGSP6GEN once, immediately adjacent and downstream of the 25

nucleotide long poly dA:dT stretch. The linearized pVGSP6GEN clone is purified with GeneClean II™ and adjusted to a concentration of 0.5 mg/ml. Transcription of the linearized pVGSP6GEN clone is performed *in vitro* at 37_C for 90 minutes according to the following reaction conditions: 2 µl DNA/4.25 µl H_2O; 10 µl 2.5 mM NTPs (UTP, ATP, GTP, CTP); 1.25 µl 20 mM $Me^7G(5')ppp(5')G$ cap analogue; 1.25 µl 100 mM DTT; 5 µl 5X transcription buffer (Promega, Madison, WI); 0.5 µl RNasin (Promega, Madison, WI); 0.25 µl 10 mg/ml bovine serum albumin; and 0.5 µl SP6 RNA polymerase (Promega, Madison, WI). The *in vitro* transcription reaction products are subsequently digested with DNase I (Promega, Madison, WI) and purified by sequential phenol/$CHCl_3$ and ether extraction, followed by ethanol precipitation, or alternatively, are used directly for transfection. The *in vitro* transcription reaction products or purified RNA are complexed with a commercial cationic lipid compound (Lipofectin™, GIBCO-BRL, Gaithersburg, MD), and applied to BHK-21 cells maintained in a 60 mM petri dish at 75% confluency. The transfected cells are incubated at 30_C. After 94 hours post-transfection, extensive cytopathic effect (cpe) are observed. No obvious cpe is observed in plates not receiving RNA transcribed from the Sindbis cDNA clone. Further, 1.0 ml of supernatant taken from transfected cells, added to fresh monolayers of BHK-21 cells, and incubated at 30_C or 37_C results in obvious cpe within 18 hours. This demonstrates that the Sindbis cDNA clone pVGSP6GEN is indeed infectious.

Sequence analysis of pVGSP6GEN, shown in Table 1, reveals multiple sequence differences between the Sindbis genomic clone described herein and the viral clone whose sequence is contained in Genbank. Many sequence differences result in non-conservative amino acids changes in the Sindbis proteins. To address which sequence changes are unique to the clone described herein, or result from cloning artifacts, virion RNA is amplified by RT-PCR as described above, and sequence relating to the nucleotides in question is determined by direct sequencing of the RT-PCR amplicon product using a commercially available kit (Promega, Madison, WI) and compared to the corresponding pVGSP6GEN sequence. The results of this study are given in Table 2. Briefly, three non-conservative amino acid changes, Gly to Glu, Asp to Gly, and Tyr to Cys, which are a result of cloning artifacts, are observed at viral nucleotides 2,245, 6,193, and 6,730, respectively. These nt changes result in non-conservative amino acid substitutions which all map to the viral non-structural protein (NSP) genes, nt 2,245 to NSP 2, and nt 6,193 and 6,730 to NSP4.

Repair of the NSP 2 and NSP 4 genes is accomplished by RT-PCR, as described above, using virion RNA from a 5 times plaque purified stock. The SP6-1A/1B primer pair described above is used to repair the nt 2,245 change. The RT-PCR amplicon product is digested with Eco 47III and Bgl II, and the 882 bp fragment is purified by 1% agarose/TBE

gel electrophoresis, and inserted into the corresponding region of the pVGSP6GEN clone, prepared by digestion with Eco 47III and Bgl II, and treatment with CIAP. The 3A/7349R primer pair described above is used to repair the nt 6,193 and nt 6,730 changes. The RT-PCR amplicon product is digested with Eco RI and Hpa I, and the 1,050 bp fragment is purified by 1% agarose/TBE gel electrophoresis, and exchanged with the corresponding region of the pVGSP6GEN clone. This clone is designated pVGSP6GENrep. Transfection of BHK-21 cells with *in vitro* transcribed RNA from pVGSP6GENrep DNA linearized by digestion with Xba I results in extensive cpe 18 hours post transfection.

Table 1

Sindbis Genomic Clone Differences between Viagene and GenBank Sequences

SIN nt No.	Change	Codon Change	Location in Codon	Amino Acid Change
Noncoding Region:				
45	T->C	N.A.	N.A.	N.A.
Non-structural Proteins:				
353	C->T	UAU->UAC	3'	Tyr->Tyr
1,095	A->C	AUA->CUA	1'	Ile->Leu
1,412	T->C	UUU->UUC	3'.	Phe->Phe
2,032	A->G	GAG->GGG	2'	Glu->Gly
2,245	G->A	GGG->GAG	2'	Gly->Glu
2,258	A->C	UCA->UCC	3'	Ser->Ser
2,873	A->G	CAA->CAG	3'	Gln->Gln
2,992	C->T	CCC->CUC	2'	Pro->Leu
3,544	T->C	GUC->GCC	2'	Val->Ala
3,579	A->G	AAA->GAA	1'	Lys->Glu
3,822	A->G	ACC->GCC	1'	Thr->Ala
3,851	T->C	CUU->CUC	3'	Leu->Leu
5,351	A->T	CAA->CAU	3'	Gln->His
5,466	G->A	GGU->AGU	1'	Gly->Ser
5,495	T->C	AUU->AUC	3'	Ile->Ile
5,543	A->T	ACA->ACU	3'	Thr->Thr
5,614	T->C	GUA->GCA	2'	Val->Ala
6,193	A->G	GAC->GGC	2'	Asp->Gly
6,564	G->A	GCA->ACA	1'	Ala->Thr
6,730	A->G	UAC->UGC	2'	Tyr->Cys
Structural Proteins:				
8,637	A->G	AUU->GUU	1'	Ile->Val
8,698	T->A	GUA->GAA	2'	Val->Glu
9,108	AAG del	AAG->del	1'-3'	Glu->del
9,144	A->G	AGA->GGA	1'	Arg->Gly
9,420	A->G	AGU->GGU	1'	Ser->Gly
9,983	T->G	GCU->GCG	3'	Ala->Ala
10,469	T->A	AUU->AUA	3'	Ile->Ile
10,664	T->C	UUU->UUC	3'	Phe->Phe
10,773	T->G	UCA->GCA	1'	Ser->Ala

Table 2

Sindbis Genomic Clone Artifact Analysis

SIN nt No.	Amino Acid Change	Viagene Unique	Cloning Artifact
Nonstructural Proteins:			
2,032	Glu->Gly	+*	
2,245	Gly->Glu		+
2,258	Ser->Ser	+*	
2,873	Gln->Gln	+	
2,992	Pro->Leu	+	
3,544	Val->Ala	.	+
3,579	Lys->Glu	+	
3,822	Thr->Ala		+
3,851	Leu->Leu		+
5,351	Gln->His	+	
5,466	Gly->Ser		+
5,495	Ile->Ile		+
5,543	Thr->Thr		+
6,193	Asp->Gly		+
6,730	Tyr->Cys		+
Structural Proteins:			
8,637	Ile->Val	+	
8,698	Val->Glu	+	
9,108	Glu->del	+	
9,144	Arg->Gly	+	

* Mixture: Both Genbank and Viagene Sindbis strains present at this nucleotide.

ii.   Generation of DNA Vectors Which Initiate Alphavirus Infection: Eukaryotic Layered Vector Initiation Systems

As noted above, the present invention provides eukaryotic layered vector initiation systems which generally comprise a promoter which is capable of initiating the 5' synthesis of RNA from cDNA, a construct which is capable of autonomous or autocatalytic replication in a cell, the construct also being capable of expressing a heterologous nucleic acid sequence(s), and a 3' sequence which controls transcription termination. Within one

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embodiment, such constructs may be constructed of the following ordered elements: a 5'
eukaryotic promoter capable of initiating the synthesis of viral RNA at the authentic
alphavirus 5' end, a 5' sequence which is capable of initiating transcription of an alphavirus,
a nucleotide sequence encoding alphavirus nonstructural proteins, a viral junction region, a
5   heterologous sequence, an alphavirus RNA polymerase recognition sequence, and a 3'
transcription termination/polyadenylation signal sequence.   Such alphavirus cDNA
expression vectors also may include intervening sequences (introns), which are spliced from
the pre-RNA in the nucleus prior to transport to the cytoplasm, and which may improve the
overall efficiency of the system, in terms of molecules of functional mRNA transported to
10   the cytoplasm.   The intron splicing signals are located, for example, between Sindbis and
heterologous gene regions.

Construction of a eukaryotic layered vector initiation system utilizing the Sindbis
clone pVGSP6GENrep and mammalian RNA polymerase II promoters may be
accomplished essentially as follows. Briefly, plasmid pVGSP6GENrep is digested with Bgl
15   II and Xba I, and the reaction products are electrophoresed in a 0.8% agarose/TBE gel.
The resulting 9,438 bp fragment is excised, purified with GeneClean II™ and ligated into
the 4,475 bp vector fragment resulting from treatment of pCDNA3 (Invitrogen, San Diego,
CA) with Bgl II, Xba I, and CIAP. This construction is designated as pcDNASINbgl/xba.

The U3 region of the LTR from MoMLV is positioned at the 5' viral end such that
20   the first transcribed nucleotide is a single G residue, which is capped *in vivo*, followed by
the Sindbis 5' end.   Juxtaposition of the MoMLV LTR and the Sindbis 5' end is
accomplished by overlapping PCR as described below. Amplification of the MoMLV LTR
in the first primary PCR reaction is accomplished in a reaction containing the BAG vector
(Price *et al.*, *PNAS 84*:156-160, 1987) and the following primer pair:

25

Forward primer: BAGBgl2F1 (buffer sequence/Bgl II recoginition sequence/MoMLV LTR
nt 1 to 22):

(Sequence ID No.: 17)

30

        5'-TATATAGATCTAATGAAAGACCCCACCTGTAGG-3'

Reverse primer: BAGwt441R2 (SIN nt 5 to 1/MoMLV LTR nt 441 to 406):

35   (Sequence ID No.: 18)

        5'-TCAATCCCCGAGTGAGGGGTTGTGGGCTCTTTTATTGAGC-3'

PCR amplification of the MoMLV LTR with the primer pair shown above is performed using the Thermalase thermostable DNA polymerase and the buffer containing 1.5 mM MgCl$_2$, provided by the supplier. The PCR reaction, containing 5% DMSO and Hot Start Wax beads, is conducted using the following PCR amplification protocol:

Temperature (C)	Time (Min.)	No. Cycles
94	2.0	1
94	0.5	
55	0.5	35
72	0.5	
72	10.0	1

Amplification of the Sindbis 5' end in the second primary PCR reaction is accomplished in a reaction containing the pVGSP6GENrep clone and the following primer pair:

Forward primer: (MoMLV LTR nt 421 to 441/SIN nt 1 to 16):

(Sequence ID No.: 19)

    5'-CCACAACCCCTCACTCGGGGATTGACGGCGTAGTAC-3'

Reverse primer: (SIN nt 3,182 to 3,160):

(Sequence ID No.: 20)

    5'-CTGGCAACCGGTAAGTACGATAC-3'

PCR amplification of the MoMLV LTR is with the primer pair and amplification reaction conditions described above, utilizing the PCR amplification protocol shown below:

Temperature (°C)	Time (Min.)	No. Cycles
94	2.0	1
94	0.5	
55	0.5	35
72	3.0	
72	10.0	1

The 457 bp and 3,202 bp products from the primary PCR reactions are purified with GeneClean II™, and used together in a PCR reaction with the following primer pair:

5   Forward primer: BAGBgl2F1 (buffer sequence/Bgl II recoginition sequence/MoMLV LTR nt 1 to 22):

(Sequence ID No.: 21)

10   5'-TATATAGATCTAATGAAAGACCCCACCTGTAGG-3'

Reverse primer: (SIN nt 2,300 to 2,278):

(Sequence ID No.: 22)

15
          5'-GGTAACAAGATCTCGTGCCGTG-3'

PCR amplification of the primer PCR amplicon products is with the primer pair and amplification reaction conditions described above, utilizing the following PCR amplification
20   protocol shown below:

Temperature (°C)	Time (Min.)	No. Cycles
94	2.0	1
94	0.5	
55	0.5	35
72	3.0	
72	10.0	1

The 25 3' terminal bases of the first primary PCR amplicon product overlap with the 25 5' terminal bases of the second primary PCR amplicon product; the resultant 2,752 bp

overlapping secondary PCR amplicon product is purified by 0.8% agarose/TBE electrophoresis, digested with Bgl II, and the 2,734 bp product is ligated into pcDNASINbgl/xba treated with Bgl II and calf intestine alkaline phosphatase (CIAP). The resulting construction is 16,656 bp and is designated pVGELVIS. Sindbis nucleotides are
5 contained within bases 1 to 11,700 of the sequence.

pVGELVIS plasmid DNA is complexed with Lipofectamine according to the conditions suggested by the supplier (ca. 5 µg DNA/8 µg lipid reagent) and added to 35 mm wells containing BHK-21 cells at approximately 75% confluency. Cytopathic effects, characteristic of wild type Sindbis virus infection are observed within 48 hours post
10 infection. Addition of 1.0 ml of transfection supernatant to fresh BHK-21 monolayers results in cpe within 16 hours. This data demonstrates the correct juxtaposition of the viral cDNA and RNA polymerase II expression cassette signals in the pVGELVIS construct result in the *de novo* initiation of an RNA virus from a DNA expression module.

In order to determine the relative efficiency of the pVGELVIS plasmid DNA to
15 initiate infection characteristic of wild-type Sindbis virus after transfection into BHK cells, an infectious centers assay is performed. Briefly, 5 µg of pVGELVIS plasmid DNA is transfected onto BHK-21 cells in 35 mm wells as described above, and at 1.5 hours post-transfection the cells are trypsinized and serially diluted 10,000-fold, over 10-fold increments, into 5×10^5 untreated BHK-21 cells. This transfected and untreated BHK-21
20 cell mixture is then added to 35 mm wells. The cells are allowed to attach to the plate, and subsequently overlayed with media containing 1.0% Noble agar. At 48 hours post-transfection, plaques due to cell lysis (as a result of Sindbis virus replication) may be visualized either directly or after overlaying with a second layer containing neutral red stain. This experiment reveals that the efficiency of the pVGELVIS plasmid in generating wild
25 type Sindbis virus after transfection onto BHK-21 cells is approximately 1.0×10^3 pfu/µg of plasmid DNA.

iii.   Construction of the Sindbis RNA and Plasmid DNA Basic Vector

30 A first step in the construction of the Sindbis Basic Vector is the generation of two plasmid subclones containing separate elements from the viral 5' and 3' ends. These elements may then be utilized in order to subsequently assemble a basic gene transfer vector.

Briefly, the first plasmid subclone is constructed to contain the 40 terminal
35 nucleotides of the viral 3' end and a 25 bp stretch of consecutive dA:dT nucleotides. In particular, the following oligonucleotide pairs are first synthesized:

- 50 -

Forward Primer: SIN11664F: (buffer sequence/Not I site/ SIN nt 11,664 to 11,698):

(Sequence ID No.: 23)

5   5'-TATATGCGGCCGCTTTCTTTTATTAATCAACAAAATTTTGTTTTTAA-3'

Reverse Primer: SIN*Sac*11700R (buffer sequence/Sac I site dT25/SIN nt 11,700 to 11,692):

(Sequence ID No.: 24)

10

        5'-TATATGAGCTCTTTTTTTTTTTTTTTTTTTTTTTTTTTGAAATGTTAAAA-3'

        The above oligonucleotides are then mixed together at equal molar concentrations
in the presence of 10 mM MgCl₂, heated to 100_C for 5 minutes and cooled slowly to
15   room temperature. The partially double-stranded molecule is then filled in using Klenow
DNA polymerase and 50 µM dNTPs. The resultant 89 bp molecule is then digested with
Not I and Sac I, purified on a 2% NuSieve/1% agarose gel, and ligated into pKS II+
plasmid, prepared by digestion with Not I and Sac I and treatment with CIAP, at a 10:1
molar excess of insert to vector. This construction is designated pKSII3'SIN.

20   The second plasmid subclone is constructed to contain the first 5' 7,643 nucleotides
of Sindbis and a bacteriophage RNA polymerase promoter positioned at the viral 5' end
such that only a single non-viral nucleotide is added to the authentic viral 5' end after
in vitro transcription.   Briefly, the 3' end of this clone is derived by a standard three
temperature PCR amplification with a reverse primer having the sequence shown below.

25

Reverse Primer: SINXho7643R (buffer sequence/Xho I site/SIN nt 7,643 to 7,621):

(Sequence ID No.: 25)

30   5'TATATCTCGAGGGTGGTGTTGTAGTATTAGTCAG-3'

        The reverse primer maps to viral nucleotides 7,643 to 7,621 and is 41 bp
downstream from the junction core element 3' end. Additionally, viral nucleotide 7,643 is 4
nucleotides upstream from the structural protein gene translation initiation codon. The first
35   five 5' nucleotides in this primer are included to serve as a 'buffer sequence' for the efficient
digestion of the PCR amplicon products, and are followed by 6 nucleotides comprising the
Xho I recognition sequence.

The forward primer in this reaction is primer 2A (*see* Example 1C(i)), having the following sequence:

(Sequence ID No.: 8)

5'-ATACTAGCCACGGCCGGTATC-3'

The 4,510 bp amplicon product, resulting from the PCR amplification shown above with pVGSP6GENrep plasmid (*see* Example 1C(i)) as template, is digested with the enzymes Sfi I and Xho I. The resultant 2,526 bp fragment is gel purified. Sindbis cDNA clone pVGSP6GENrep is also digested with Apa I and Sfi I, and the resultant 5,144 bp fragment which includes the SP6 RNA polymerase promoter at its 5' end is gel purified. The 5,144 bp fragment is ligated together with the 2,526 bp fragment from above, along with the Apa I and Xho I digested, CIAP treated pKS II+ plasmid. A clone is isolated having the Sindbis nucleotides 1 to 7,643 and including an RNA polymerase promoter at its 5' end contained in the pKSII+ plasmid vector. This construction is designated pKSII5'SIN.

Assembly of the complete basic vector is accomplished by digesting pKSII5'SIN with Xho I and Sac I, treating with CIAP, and gel purifying a large 10,533 bp fragment. The 10,533 bp fragment is then ligated together with a 168 bp small fragment resulting from digestion of pKSII3'SIN with Xho I and Sac I. This resultant construction is designated pKSSINBV, and is shown in Figure 11A.

iv.   Construction of Sindbis Luciferase Vector

The firefly luciferase reporter gene is inserted into the Sindbis Basic Vector in order to demonstrate the expression of a heterologous gene in cells transfected with RNA that is transcribed *in vitro* from the Sindbis vector clone, and to demonstrate the overall functionality of the Sindbis basic vector.

Construction of the Sindbis luciferase vector is performed by assembling together components of 3 independent plasmids: pKSII5'SIN, pKSII3'SIN, and pGL2-basic vector. The pGL2-basic vector plasmid (Promega, Madison, WI) contains the entire firefly luciferase gene. Briefly, the luciferase gene is first inserted into the pKSII3'SIN plasmid. This is accomplished by digesting pGL2 with Bam HI and Hind III and gel purifying a 2,689 bp containing fragment. This fragment is ligated with a gel purified 3,008 bp

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fragment resulting from digestion of pKSII3'SIN with Bam HI and Hind III and treatment with CIAP. The resultant construction is designated pKSII3'SIN-luc.

Final assembly of a Sindbis luciferase vector is accomplished by digesting pKSII5'SIN with Xho I and Sac I, treating with CIAP, and gel purifying the large 10,533

5   bp fragment. The pKS 5'SIN 10,533 bp fragment is ligated together with the 2,854 bp small fragment resulting from digestion of pKSII3'SIN-luc with Xho I and Sac I. This construction contains the entire Sindbis nonstructural gene coding region and 3' viral elements necessary for genome replication, as well as the firefly luciferase gene positioned between these two viral 5' and 3' elements. This vector is designated pKSSINBV-luc, and

10 is shown schematically in Figure 11B.

v.   Construction Of Plasmid DNA Alphavirus Expression Vectors

The SIN BV and SIN-BV-luciferase constructs described in Section D(iii), above,

15 are inserted into the pVGELVIS vector configurations described in Section D(ii), such that expression of the heterologous gene from Sindbis vectors occurs after direct introduction of the plasmid DNA into cells. As described in Section D(ii), the ability to transfect alphavirus-based vector plasmid DNA directly into cells and produce expression levels of heterologous genes typical of transfection of RNA-based alphavirus vectors without a

20 primary step consisting of *in vitro* transcription of linearized template vector DNA enhances greatly the utility and efficiency of certain embodiments of the alphavirus-based expression vector system. Figure 12 is a schematic representation of one mechanism of expression of heterologous genes from a plasmid DNA alphavirus expression (ELVIS) vectors. Primary transcription in the nucleus and transport of the vector RNA to the

25 cytoplasm leads to the synthesis of alphavirus nonstructural proteins which catalyze the expansion of heterologous gene mRNA via an antigenome intermediate which in turn serves as the template for production of genomic and subgenomic mRNA. The ELVIS vectors may be introduced into the target cells directly by physical means as a DNA molecule, as a complex with various liposome formulations, or as a DNA-ligand complex

30 including the alphavirus DNA vector molecule, a polycation compound such as polylysine, a receptor specific ligand, and, optionally, a psoralen inactivated virus such as Sendai or Adenovirus.

The first step of constructing a representative plasmid DNA Sindbis expression vector consists of digesting pKSSINBV with Sac I, blunting with T4 polymerase, digesting

35 with Sfi I, isolating the 2,689 bp fragment, and ligating into the pVGELVIS 10,053 bp vector fragment prepared by digestion with XbaI, blunting with T4 polymerase, digesting

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with Sfi I, treatment with CIAP, and 1% agarose/TBE gel electrophoresis. This construction is designated pVGELVIS-SINBV.

In order to insert the luciferase gene into the pVGELVIS-SINBV vector, the SV40 intron and transcription termination sequences at the 3' end of luciferase must be removed
5   so that when the pre-RNA, transcribed from the plasmid DNA luciferase vector after transfection into cells, is processed the 3' end of the reporter gene is not separated from the Sindbis vector 3' end, as the Sindbis 5' and 3' ends contained within the pVGELVIS-SINBV vector are required in *cis* for the autocatalytic replication activity of the vector. The Sindbis vector 3' end is required for initiation of synthesis of the antigenomic strand,
10 which is the template for the subgenomic RNA encoding the heterologous or reporter protein.

The SV40 RNA processing signals positioned at the 3' end of the luciferase gene are removed from the SIN-BV-luc construction described in Section D(iv) above. The modified luciferase fragment is then placed in the pVGELVIS-SINBV construction
15 described above via unique restriction sites. The alteration of the luciferase gene is accomplished with the primer pair shown below:

Forward primer 7328F (SIN nt 7,328 to 7,349):

20 (Sequence ID No.: 12)

5'-GTGGCGGATCCCCTGAAAAGG-3'

Reverse primer LucStop (buffer sequence/Not I, Xba I recognition sequences/pGL-2 nt
25 1,725 to 1,703):

(Sequence ID No.: 26)

5'-TATATGCGGCCGCTCTAGATTACAATTTGGACTTTCCGCCC-3'

30
The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period. The amplification products are purified with GeneClean II™, digested with Xho I and Xba I, purified again with GeneClean II™, and the 2,037 bp fragment is ligated into the 13,799 bp fragment of pVGELVIS-SINBV
35 resulting from digestion with Xho I and Xba I, and treatment with CIAP. This construction is designated pVGELVIS-SINBV-luc (abbreviated as ELVIS-luc).

The expression of luciferase in BHK-21 cells transfected with pVGELVIS-SINBV-luc DNA is measured in order to demonstrate that the Sindbis physical gene transfer vector is functional. Briefly, 5 μg of pVGELVIS-SINBV-luc DNA or 5 μg of *in vitro* transcribed RNA from linearized SINBV-luc template as described in Section D(iv), above, are complexed with 10 μl of Lipofectamine™ or Lipofectin™, respectively, and transfected into 5 x 10⁵ BHK-21 cells contained in 35 mM petri plates. The luciferase activity is determined from each of three samples at 2, 4, 8, 16, 20, 28, 48, 72, 96, and 120 hours post transfection. The results of this study, given in Figure 13, demonstrate that the maximal level of reporter gene expression from the pVGELVIS-SINBV-luc vector is similar to that observed in cells transfected with *in vitro* transcribed RNA from linearized SINBV-luc template. However, the luciferase activity expressed from the pVGELVIS-SINBV-luc vector is at maximal levels at later time points compared to that observed with the SINBV-luc RNA vector, and continues at high levels while the activity from the RNA vector begins to diminish.

The overall efficiency of the ELVIS vector, as determined by level of heterologous gene expression, is enhanced by several modifications to the pVGELVIS-SINBV-luc vector, as described in detail in (U.S.S.N. 08/348,472). These modifications include alternate RNA polymerase II promoters and transcription termination signals, additions of intron sequences in the vector construct, insertion of ribozyme processing sequences adjacent to the alphavirus 3'-end, and substitution with a smaller plasmid vector.

A linker sequence also is inserted into the pKSSINBV and pVGELVIS-SINBV constructs to facilitate the insertion of heterologous sequences. The linker is constructed using two complementary oligonucleotides that form a duplex with Xho I and Xba I compatible sticky ends when hybridized.

SINBVLinkF:

(Sequence ID No.: 27)

5'-TCGAGCACGTGTTTAAACGCGTGATCAGGCCT-3'

SINBVLinkR:

(Sequence ID No.: 28)

5'-CTAGAGGCCTGATCACGCGTTTAAACACGTGC-3'

The oligonucleotides are phosphorylated with T4 polynucleotide kinase, heated to 90_C, and slow-cooled to allow hybridization to occur. The hybrid is then ligated to the 10.6 kb fragment of pKSSINBV-Luc obtained after digestion with Xho I and Xba I, followed by treatment with alkaline phosphatase and agarose gel purification. The resulting construct

5   contains Xho I, Pml I, Pme I, Mlu I, Bcl I, Stu I, Xba I, and Not I as unique sites between the Sindbis junction region and the Sindbis 3' end. This construct is designated pKSSINBVLII.

The polylinker also is cloned into the pVGELVIS-SINBV constructs. The linker is inserted by digestion of pVGELVIS-SINBV-luc with Sfi I and Not I. The 10.1kb fragment

10 is agarose gel purified, and this fragment was ligated to the gel purified 2.6kb fragment from a Sfi I/Not I digest of pKSSINBVLII. The resulting construct contains Xho I, Pml I, Pme I, Mlu I, and Not I as unique sites between the Sindbis junction region and the Sindbis 3' end. This construct is designated pVGELVIS-SINBVLII.

Plasmid pKSSINBVLII is modified further by the addition of an Asc I eight

15 nucleotide recognition sequence adjacent to the Sac I transcription run-off site. The relative infrequency of this recognition sequence allows for linearization of a wide range of heterologous gene-containing Sindbis vector constructs. Specifically, an oligonucleotide, which has Sac I compatible termini when self-annealed, is used.

20 AscI/SacI linker:

(Sequence ID No.: 29)

        5'-CGGCGCGCCGAGCT-3'

25

The AscI/SacI linker is phosphorylated with T4 polynucleotide kinase, heated to 90_C, and slow-cooled to allow self-annealing to occur. The duplex is then ligated into pKSSINBVLII plasmid DNA which has been digested with Sac I and treated with calf intestinal alkaline phosphatase. The resulting construct is designated pKSSINBVLIIA.

30

D.   Generation of Recombinant Sindbis CEA Vectors

    i.   Construction of a Recombinant Sindbis Vector (SIN-CEA) Dependent on the
            Expression of the CEA Tumor Marker

35

As described previously and shown diagrammatically in Figure 14, the disabled junction loop out model is constructed with the junction region of the vector flanked by

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inverted repeat sequences which are homologous to the RNA of choice. In this example, sequences from the CEA tumor antigen cDNA (Beauchemin *et al.*, *Molec. and Cell. Biol.* 7:3221, 1987) are used in the inverted repeats. To construct a CEA RNA responsive Sindbis vector, the junction region is preceded by two CEA anti-sense sequence domains

5 (A¹ and B¹) separated by a six bp hinge domain. A single twenty base pair CEA sense sequence (A2), which is complementary to A1, is placed at the 3' end of the junction region. In choosing the correct A1 and B1 antisense sequences, the only two requirements are that they be specific for the targeted RNA sequence and that the anti-sense sequences hybridize to two RNA sequence domains separated by three nucleotides. This three

10 nucleotide gap will serve as a hinge domain for the polymerase to hop and switch reading strands bridging the non-structural protein domain of the vector to the junction region of the vector (Figure 15). To construct such a configuration, two oligonucleotides are synthesized complementing each other to create a fragment insert containing convenient restriction enzyme sites at the extreme 5' and 3' ends. The oligonucleotide fragment insert

15 is then ligated into the Sindbis vector between the disabled junction region and the multiple cloning sites of the Sindbis vector. The sense oligonucleotide strand, from 5' to 3', should contain an Apa I restriction site, followed by the A1 anti-sense domain, a six bp hinge domain, a B1 anti-sense domain, a synthetic junction region domain, and the A2 sense domain, followed by a Xho I restriction enzyme site. The following oligonucleotide

20 sequence is used to design a CEA RNA responsive Sindbis vector. The nucleotide number sequence is obtained from Beauchemin *et al.*, *Molec. and Cell Biol.* 7:3221, 1987.

5'-3' CEA sense strand:
(Sequence ID No.: 30)

25   CEA 618   CEA 589
              Apa I   *─────────────────────────────────────*
5'-CGC GC   G GGC   CCT GT   G ACA T   TG AAT   AGA GT   G AGG G   TC CTG
TTG GG-3'

30
(Sequence ID No.: 31)

              CEA 651   CEA 622
              ───   *

35 Synthetic
5'-A AAG G   TT TCA   CAT TT   G TAG C   TT GCT   GTG TC   A TTG C   GA TCT
CTA CG-3'

(Sequence ID No.: 32)

```
                        CEA 599              CEA 618
    Junction Core       •      •_____•      Xho I
5   5'-G TGG T  CC TAA  ATA GT  T CAC T  CT ATT  CAA TG  T CAC A  CT CGA
    GCC GG-3'
```

The 5'-3' CEA anti-sense strand is complementary to the above oligonucleotide. After both oligonucleotides are synthesized, the oligonucleotides are mixed together in the
10 presence of 10 mM Mg⁺² heated to 100_C for 5 minutes and cooled slowly to room temperature. The oligonucleotide pair is then digested with the Apa I and Xho I restriction enzymes, mixed and ligated at a 25:1 molar ratio of insert to plasmid (pCMV-SIN or pMET-SIN) predigested with the same enzymes. These constructs are designated pCMV/SIN-CEA and pMET/SIN-CEA, respectively.

15

    ii.   Construction of a SIN-CEA Vector and Producer Cell Line Expressing γ Interferon (SIN-CEA/IFN)

The human gamma interferon (γ-IFN) gene is subcloned from the retroviral vector
20 plasmid pHu-IFN (Howard et al., Ann N.Y. Acad. Sci. 716:167-187, 1994) by digesting with Xho I and Cla I. The resulting 500 bp fragment containing the coding sequences of γ-IFN is isolated from a 1% agarose gel.

Alternatively, the human γ-IFN cDNA is derived from RNA isolated from PHA-stimulated Jurkat T cells by guanidinium thiocyanate extraction followed by
25 ultracentrifugation through a CsCl gradient. The RNA (Sigma, St. Louis, MO) is then reverse-transcribed in vitro and a gene-specific oligonucleotide pair is used to amplify γ-IFN cDNA by polymerase chain reaction using Taq polymerase. The PCR DNA was repaired with T4 DNA polymerase and Klenow and cloned into the Hinc II site of pSK⁺ plasmid treated with CIAP. In the sense orientation, the 5' end of the cDNA is adjacent to
30 the Xho I site of the pSK⁺ polylinker and the 3' end adjacent to the Cla I site. The 512 base pair fragment encoding the human γ-IFN molecule is placed into the Xho I/Cla I site of either the pCMV/SIN-CEA or pMET/SIN-CEA vectors. These new plasmids are designated pCMV/SIN-CEA/IFN or pMET/SIN-CEA/IFN, respectively.

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iii.   <u>Construction of a SIN-CEA Vector Expressing Thymidine Kinase (SIN-CEA/TK)</u>

A PCR amplified product containing the cDNA clone of the HSVTK, flanked with
5   5' Xho I and 3' Cla I restriction enzyme sites is obtained using the pHS1TK3KB (McKnight
et al., *Nuc. Acids Res.* 8:5949, 1980) clone as target DNA. The sequences for the primers
used for the PCR amplification are obtained from published sequences (Wagner *et al.*,
PNAS 78:1442, 1981). The 1,260 bp amplified product is then digested with Xho I and Cla
I ligated into the Xho I / Cla I site of either the pCMV/SIN-CEA or pMET/SIN-CEA
10   vectors. These new plasmids are designated pCMV/SIN-CEA/HSVTK or pMET/SIN-
CEA/HSVTK, respectively.

E.   <u>Construction of Recombinant Vectors Containing Human Tissue Factor Gene</u>
15

Specifically, the cloning of a human tissue factor gene into recombinant retroviral
vectors and Sindbis vectors is accomplished by PCR amplification using two 38 base
oligonucleotides. The oligonucleotides contain 25 nucleotides corresponding to the tissue
factor gene template, plus additional 5'-flanking sequences comprising a Pme I recognition
20   site and buffer sequence, as follows.

HTFP-F:

(Sequence ID No.: 33)
25

5'-TATATGTTTAAACCCGCCAACTGGTAGACATGGAGACC-3'

HTFP-R:

30   (Sequence ID No.: 34)

5'-ATATAGTTTAAACTCCAACAGTGCTTCCTTTATGAAAC-3'

PCR amplification using these primers, tissue factor cDNA clone λHTF7 (Scarpati *et al.*,
35   *Biochemistry* 26:5234, 1987) as template, Thermalase thermostable DNA polymerase, 1.5
mM $MgCl_2$ provided by the supplier, 5% DMSO, and Hot Start Wax beads, is performed
according to the following protocol:

Temperature	Time (Min.)	No. Cycles
94	2.0	1
94	0.5	
50	0.5	5
72	2.0	
94	0.5	
55	0.5	25
72	2.0	
72	10.0	1

Following amplification, the approximately 920 bp tissue factor gene amplicon is digested with *Pme I* and ligated into Sindbis vector (pVGELVIS-SINBVLII or pKSSINBVLIIA), or retroviral vector backbones (pKT-1L, pKT-3BL, or pKT-3BCL) that have been digested
5 with *Pme I* and treated with CIAP. Proper orientation of the insert sequence is determined by restriction endonuclease digests and the resulting constructs are designated pVGELVIS-HTF, pKSIN-HTF, pKT-1L-HTF, pKT-3BL-HTF, and pKT-3BCL-HTF, respectively. Production of packaged vector particles is accomplished using the methods described in Example 2 below. DNA encoding other gene products useful in the practice of this
10 invention may be readily cloned into these or other vectors using these approaches.

F.   Construction of Recombinant Vectors Containing Tissue Inhibitor of Metalloproteinase-I

15   Specifically, the cloning of a human TIMP-1 gene into recombinant retroviral vectors and Sindbis vectors is accomplished by PCR amplification using two 38-mer oligonucleotides. The oligonucleotides contain 25 nt corresponding to the TIMP-1 gene template, plus additional 5'-flanking sequences comprising a Pme I recognition site and buffer sequence, as follows:

20

HTIMP-F:

(Sequence ID No.: 35)

25   5'-TATATGTTTAAACCACCAGAGAACCCACCATGGCCCCC-3'

HTIMP-R:

(Sequence ID No.: 36)

5   5'-ATATAGTTTAAACCCACTCCGGGCAGGATTCAGGCTAT-3'

PCR amplification using these primers, a TIMP-1 cDNA clone (Docherty *et al.*, *Nature* *318*:66, 1985) as template and Thermalase thermostable DNA polymerase, is performed in a reaction containing 1.5 mM MgCl$_2$ provided by the supplier, 5% DMSO, and Hot Start

10 Wax beads according to the following protocol:

Temperature	Time (Min.)	No. Cycles
94	2.0	1
94	0.5	
50	0.5	5
72	2.0	
94	0.5	
55	0.5	25
72	2.0	
72	10.0	1

Following amplification, the approximately 700 bp TIMP-I gene amplicon is digested with Pme I and ligated into Sindbis vector (pVGELVIS-SINBVLII or pKSSINBVLIIA), or

15 retroviral vector backbones (pKT-1L, pKT-3BL, or pKT-3BCL) that have been digested with Pme I and treated with CIAP. Proper orientation of the insert sequence is determined by restriction endonuclease digests and the resulting constructs are designated pVGELVIS-HTIMP, pKSIN-HTIMP, pKT-1L-HTIMP, pKT-3BL-HTIMP, and pKT-3BCL-HTIMP, respectively. Production of packaged vector particles is accomplished using the methods

20 described in Example 2 below. Other related gene products from this group are readily cloned using these approaches and oligonucleotide primers that contain Pme I or other appropriate restriction sites, and are specific for their respective gene.

G.   Construction of Recombinant Vectors Containing Recombinant Folate Receptor
      Containing Extracellular Matrix Binding Fragment Derived from Integrin β3 into
      KT-3

5   i.   Construction of Folate Receptor/Integrin β3 Fusion Gene Via Amino
            Terminus Glycine Tether Linkage

      The cloning of a sequence encoding the human folate receptor type α with the
ligand binding domain of integrin β3 linked to it's amino terminus by a glycine tether is
10 accomplished by overlapping PCR amplification. The two pairs of oligonucleotides used in
the primary PCR reactions produce amplicons that contain 20 overlapping bases plus
additional 5' or 3'-flanking sequences comprising a Pme I recognition site and buffer
sequence. In addition, each oligonucleotide primer contains 25·nucleotides that correspond
to sequences on the input template DNA to allow priming, as follows:

15

Folate receptor gene primers:

GHFBP-F:
(Sequence ID No.: 37)

20

      5'-GGTGGAGGTGGAAGCGCTCAGCGGATGACAACACAGCTGC-3'

HFBP-R:
(Sequence ID No.: 38)

25

      5'-ATATAGTTTAAACAGGTAAAAGGAGGTCAGCTGAGCAG-3'

PCR amplification using these primers, a human folate receptor cDNA clone, c32KB
(Elwood, et al., J. Biol. Chem. 264:14893, 1989), as template, and VENT™ DNA
30 polymerase (New England Biolabs, Beverly, MA), is performed in a reaction containing
buffer provided by the supplier, 5% DMSO, and Hot Start Wax beads according to the
following protocol:

Temperature (°C)	Time (Minutes)	Number of Cycles
94	2.0	1
94	0.5	
50	0.5	5
72	1.0	
94	0.5	
55	0.5	25
72	1.0	
72	10.0	1

Integrin β3 primers:

Iβ3109F:

5   (Sequence ID No.: 39)

5'-TATATGTTTAAACCACCATGGTCAGTCCCCAGAGGATTGCACTCC-3'

GIβ3171R:

10   (Sequence ID No.: 40)

5'-TGAGCGCTTCCACCTCCACCGAGCTTTCGCATCTGGGTGGCCTGC-3'

PCR amplification using these primers, a human integrin β3 (glycoprotein IIIa) cDNA clone

15   (Fitzgerald, *et al.*, *J. Biol. Chem.* *262*:3936, 1987) as template, and VENT™ polymerase, is performed as described above.

The folate receptor and integrin β3 PCR reactions from above are purified using GeneClean II™ and Mermaid Kit™ (BIO 101, San Diego, CA), respectively, and approximately 5% of the product from each reaction is combined and used as template in a

20   second round of PCR, containing primers Iβ3109F and HFBP-R and Thermalase DNA polymerase, as described previously and using the following amplification protocol:

Temperature (C)	Time (Minutes)	Number of Cycles
94	2.0	1
94	0.5	
50	0.5	5
72	2.0	
94	0.5	
55	0.5	25
72	2.0	
72	10.0	1

Following amplification, the approximately 1000 bp integrin β3/folate receptor gene fusion amplicon is gel purified, digested with Pme I and ligated into Sindbis vector (pVGELVIS-SINBVLII or pKSSINBVLIIA), or retroviral vector backbones (pKT-1L, pKT-3BL, or pKT-3BCL) that have been digested with Pme I and treated with CIAP. Proper orientation of the insert sequence is determined by restriction endonuclease digests and the resulting constructs are designated pVGELVIS-IF, pKSIN-IF, pKT-1L-IF, pKT-3BL-IF, and pKT-3BCL-IF, respectively.

ii.   Construction of Folate Receptor/Integrin β3 Fusion Gene by Insertion of the Ligand Binding Domain of Integrin β3 between Arg$_{227}$ and C-Terminal Hydrophobic Region

Specifically, the cloning of sequences encoding the human folate receptor type α with the ligand binding domain of integrin β3 inserted between Arg residue 227 and the C-terminal hydrophobic region is accomplished by overlapping PCR amplification. The two pairs of oligonucleotides used in the primary PCR reactions produce amplicons that contain 21 bases which overlap with each other, plus additional 5' or 3'-flanking sequences comprising a Pme I recognition site and buffer sequence. In addition, each oligonucleotide primer contains a minimum of 25 nucleotides that correspond to sequences on the input template DNA to allow priming, as follows:

Folate receptor gene primers:
HFBP-F:
(Sequence ID No.: 41)

5'-TATATGTTTAAACAGACATGGCTCAGCGGATGACAACA-3'

HFBP-R/β3:

(Sequence ID No.: 42)

    5'-TGCAATCCTCTGGGGACTGACCCTCGCCACCTCCTCATTGGGGTTG-3'

PCR amplification using these primers, a human folate receptor cDNA clone, c32KB
(Elwood, *et al.*, *J. Biol. Chem.* *264*:14893, 1989) as template, and VENT™ DNA
polymerase is performed in a reaction containing buffer provided by the supplier, 5%
DMSO, and Hot Start Wax beads according to the following protocol:

Temperature (C)	Time (Minutes)	Number of Cycles
94	2.0	1
94	0.5	
50	0.5	5
72	1.0	
94	0.5	
55	0.5	25
72	1.0	
72	10.0	1

Integrin β3 and β3/C-terminus fusion primers:

Iβ3LBD-F:

(Sequence ID No. 43)

        5'-GTCAGTCCCCAGAGGATTGCACTCC-3'

Iβ3LBD-R/FC-TERM:

(Sequence ID No. 44)

        5'- ATATAGTTTAAACTCAGCTGAGCAGCCACAG
            CAGCATTAGGGCCAGGCTAAGCAGGAAAGGCCAGGCTGCCC
            AGGGCCCAGCCCCACTCATGGCTGCAGCATAGAAGAGCTTT
            CGCATCTGGGTGGCCAGC-3'

PCR amplification using these primers (Iβ3LBD-R/FC-TERM must be FPLC purified by
the manufacturer), a human integrin β3 (glycoprotein IIIa) cDNA clone (Fitzgerald, *et al.*,

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J. Biol. Chem. 262:3936, 1987) as template, and VENT polymerase, is performed as described above.

The folate receptor and integrin β3 PCR reactions from above are purified using GENECLEAN and approximately 5% of the product from each reaction is combined and used as template in a second round of PCR, containing primers HFBP-F and Iβ3LBD-R/FC-TERM and Thermalase DNA polymerase, as described previously and using the following amplification protocol:

Temperature (C)	Time (Minutes)	Number of Cycles
94	2.0	1
94	0.5	
50	0.5	5
72	2.0	
94	0.5	
55	0.5	25
72	2.0	
72	10.0	1

Following amplification, the approximately 1000 bp integrin β3/folate receptor gene fusion amplicon is gel purified, digested with Pme I and ligated into Sindbis vector (pVGELVIS-SINBVLII or pKSSINBVLIIA), or retroviral vector backbones (pKT-1L, pKT-3BL, or pKT-3BCL) that have been digested with Pme I and treated with calf intestinal alkaline phosphatase. Proper orientation of the insert sequence is determined by restriction endonuclease digests and the resulting constructs are designated pVGELVIS-FI, pKSIN-FI, pKT-1L-FI, pKT-3BL-FI, and pKT-3BCL-FI, respectively. Production of packaged vector particles is accomplished using the methods described in Example 2.

H.   Construction of TF-IRES-PAI Retroviral Vector

   i.   Retroviral Vector with Internal Ribosome Entry Site

The plasmid pBS-ECAT (Jang, *et al.*, *J. Virol. 63*:1651, 1989) includes the 5' nontranslated region of encephalomyocarditis virus (EMCV) from nt 260-848 of the viral genome, which contains the internal ribosome entry site (IRES). EMCV nt 260-827 are amplified from pBS-ECAT by PCR using the following primer pair:

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EMCV IRES forward primer (for insertion into the MluI site in pKT-3BCL):
(Sequence ID No.: 45)

5'-TATAT - ACG CGT - CCC CCC CCC CCC CAA CG- 3'

EMCV IRES reverse primer:
(Sequence ID No.: 46)

5'-TATAT - ACG CGT - CTT ACA ATC GTG GTT TTC AAA GG-3'

The amplicon resulting from amplification with these primers is flanked by MluI recognition sites, inside a 5 bp 'buffer sequence'. Amplification of the EMCV IRES sequence from the pBS-ECAT plasmid is accomplished with the following PCR protocol:

Temperature	Time (Min.)	No. Cycles
94	2.0	1
94	0.5	
55	0.17	5
72	1.0	
94	0.5	30
70	1.0	
72	10.0	1

For insertion into the pKT-3BCL vector backbone, the IRES amplicon is digested with MluI, purified on a 1% agarose gel, and ligated into a CIAP treated vector previously digested with MluI. This construct is designated pKT-3BCL(IR). Correct orientation of the insert is determined by sequencing the resulting plasmid. In addition, this amplicon may be inserted into other retroviral vector backbones and the Sindbis vector backbones pKSSINBVL II and pVGELVIS-SINBVL II.

ii.   Retroviral Vector with Tissue Factor and Plasminogen Activator Inhibitor-1
        Genes in the pKT-3BCL(IR) Backbone

The cDNA for the human Tissue Factor protein is synthesized by PCR amplification of the cDNA from plasmid λHTF7 (Scarpati, et al., supra). The TF cDNA is amplified using the gene specific primer pair as previously described in Example 1E (HTFP-F and

HTFP-R). The amplicon resulting from amplification with these primers is flanked by Pme I recognition sites, inside a 5 bp 'buffer sequence'.

In addition to the cDNA sequences for TF protein, the primer pair contains 5 nucleotide "buffer sequences" for efficient enzyme digestion followed by the PmeI recognition sequence. For insertion into pKT-3BCL(IR), the amplicon is digested with PmeI, purified on a 1% agarose gel, and ligated into a CIAP treated vector previously digested with PmeI. This construct is designated pKT-3BCL(TF-IR). Alternatively, other restriction sites within the polylinker may be incorporated into the PCR primers for insertion into pKT-3BCL, pKSSINBVL II and pVGELVIS-SINBVL II, vectors containing IRES. Correct orientation of the insert is determined by sequencing the resulting plasmid.

The cDNA for the human plasminogen activator inhibitor -1 (PAI-1) protein is synthesized by PCR amplification of the cDNA from plasmid PAIB4 (Ginsburg, *et al.*, *J. Clin. Invest.* *78*:1673, 1986). The PAI-1 cDNA is amplified using the following gene specific primer pair. The amplicon resulting from amplification with these primers is flanked by NotI recognition sites, inside a 5 bp 'buffer sequence'.

PAI-1 cDNA forward primer:
(Sequence ID No.: 47)

        5'-TATAT - GCGGCCGC - ATG CAG ATG TCT CCA GC-3' .

PAI-1 cDNA reverse primer:
(Sequence ID No.: 48)

        5'-TATAT - GCGGCCGC - CCC AAG GTA GTG AAC CGG-3'

Amplification of the TF and PAI-1 sequences are accomplished with the following PCR protocol:

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Temperature	Time (Min.)	No. Cycles
94	2.5	1
55	0.5	1
72	1.0	
94	0.5	30
60	1.0	
72	1.0	
94	0.5	1
60	1.0	
72	5.0	

In addition to the cDNA sequences for PAI-1 protein, the primer pair contains 5 nucleotide "buffer sequences' for efficient enzyme digestion followed by the NotI recognition sequence. For insertion into pKT-3BCL(TF-IR), the amplicon is digested with 5 NotI, purified on a 1% agarose gel, and ligated into a CIAP treated vector previously digested with NotI. This construct is designated pKT-3BCL(TF-IR-PAI). Alternatively, other restriction sites within the polylinker may be incorporated into the PCR primers for insertion into pKT-3BCL, pKSSINBVL II and pVGELVIS-SINBVL II. Correct orientation of the insert is determined by sequencing the resulting plasmid(s).

Example 2

Transient Transfection and Transduction of Packaging Cell Lines HX and DA with the Vector Constructs

A.   Plasmid DNA Transfection

The following procedure can be used to transiently transfect appropriate packaging cell lines with plasmids encoding vectors according to the invention. For example, the packaging cell line HX (WO 92/05266) is seeded at 5.0×10^5 cells on a 10 cm tissue culture dish on day 1 with DMEM and 10% FBS. On day 2, the media is replaced with 5.0 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA co-precipitation is performed by mixing 40.0 µl 2.5 M $CaCl_2$, 10 µg plasmid DNA, (e.g., pkSIN-HTF, pkT-IL-HTIMP, pkT-38L (TF-IR-PAI)) and deionized H_2O in a total volume of 400 µl. The DNA-$CaCl_2$ solution is added dropwise with constant agitation to 400 µl precipitation buffer (50 mM HEPES-NaOH, pH 7.1; 0.25 M NaCl and 1.5 mM Na_2HPO_4-

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NaH$_2$PO$_4$). This mixture is incubated at room temperature for 10 minutes. The resultant fine precipitate is added to the HX of cells. The cells are incubated with the DNA precipitate overnight at 37_C. On day 3, the media is aspirated and fresh media is added. The supernatant is removed on day 4, passed through a 0.45 µl filter, and stored at -80_C.

B.   Packaging Cell Line Transduction and Generation of Producer Lines

        To increase retroviral titers produced from packaging cells, it is preferable to transduce another packaging cell line with retroviral vectors transiently produced from another cell line. For example, DA (an amphotropic cell line derived from D 17 cells ATCC No. 183, WO 92/05266) cells are seeded at 5.0 x 10^5 cells/10 cm tissue culture dish in 10 ml DMEM and 10% FBS, 4 µg/ml polybrene (Sigma, St. Louis, MO) on day 1. On day 2, 3.0 ml, 1.0 ml and 0.2 ml of the freshly collected virus-containing HX media (produced as described in Example 2(A) above) is added to the cells. The cells are incubated with the virus overnight at 37_C. In those instances when the retroviral also encodes a selectable marker, e.g., neomycin resistance, on day 3, the media is removed and 1.0 ml DMEM, 10% FBS with 800 µg/ml G418 is added to the plate. Only cells that have been transduced with the vector and expressing the selectable marker will survive. In the case of neomycin resistance, G418 resistant pools can be generated over a period of a week. Typically, a pool of cells is then dilution cloned by removing the cells from the plate and counting the cell suspension, diluting the cells suspension down to 10 cells/ml and adding 0.1 ml to each well (1 cell/well) of a 96 well plate (Corning, Corning, NY). Cells are incubated for 14 days at 37_C, 10% CO$_2$. As many as twenty-four clones are selected and expanded up to 24 well plates, 6 well plates then 10 cm plates at which time the clones are assayed for expression and the supernatant are collected and assayed for viral titer.

        The titer of the individual clones is determined by infection of HT1080 cells, (ATCC No. CCL 121). On day 1, 5.0 x 10^5 HT1080 cells are plated on each well of a 6 well microtiter plate in 3.0 ml DMEM, 10% FBS and 4 µg/ml polybrene. On day 2, the supernatant from each clone is serially diluted 10 fold and used to infect the HT1080 cells in 1.0 ml aliquots. The media is replaced with fresh DMEM, 10% FBS media, and the cells incubated with the vector overnight at 37_C, 10% CO$_2$. On day 3, selection of transduced cells is performed (assuming the presence of a selectable marker in the recombinant vector) by replacing the media with fresh DMEM, 10% FBS media containing the appropriate selection agent, for instance, 800 µg/ml G418 in the case of neomycin resistance. Cells are incubated at 37_C, 10% CO$_2$ for 14 days at which time G418 resistant colonies are scored at each dilution to determine the viral titer of each clone as cfu/ml.

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Using these procedures, cell lines are derived that produce greater than or equal to 1.0 x 10⁶ cfu/ml in culture. In addition, as those in teh art will appreciate, in those instances where selectable markers other than drug resistance, or when no selectable marker is encoded by the recombinant vector, other titer methods, such as antibody-based assays, PCR assays, etc., may be employed.

The packaging cell line HX is transduced with vector generated from the DA vector producing cell line in the same manner as described for transduction of the DA cells from HX supernatant.

Transduction of the DA or HX cells with vectors lacking a *neo* selectable marker was performed as described above. However, instead of adding G418 to the cells on day 3, the cells are cloned by limiting dilution. Titer is analyzed as described above.

C.   Alternative Method of Generation of Producer Cell Line

In some situations it may be desirable to avoid using more than one cell line in the process of generating producer lines. In this case, DA cells are seeded at 5.0 x 10⁵ cells on a 10 cm tissue culture dish on day 1 with DMEM and 10% irradiated (2.5 megarads minimum) FBS. On day 2, the media is replaced with 5.0 ml fresh media 4 hours prior to transfection. A standard calcium phosphate-DNA coprecipitation is performed by mixing 60 μl 2.0 M CaCl₂, 10 μg pMLP-G (Hartman, *et al., Nuc. Acid Res. 16*:9345, 1988; Emi, *et al., J. Vir 65*:1202, 1991) plasmid, 10 μg recombinant viral vector plasmid, and deionized water to a volume of 400 μl. The DNA-CaCl₂ solution is then added dropwise with constant agitation to 400 μl 2x precipitation buffer (50 mM HEPES-NaOH, pH 7.1, 0.25 M NaCl and 1.5 mM Na₂HPO₄-NaH₂PO₄). This mixture is incubated at room temperature for 10 minutes. The resultant fine precipitate is added to a culture dish of DA cells plated the previous day. The cells are incubated with the DNA precipitate overnight at 37_C. On day 3, the medium is removed and fresh medium is added. The supernatant containing G-pseudotyped virus is removed on day 4, passed through a 0.45 μl filter and used to infect the DA packaging cell.

DA cells are seeded at 5.0 x 10⁵ cells on a 10 cm tissue culture dish in 10 ml DMEM and 10% FBS, 4 mg/ml polybrene on day 1. On day 2, 2.0 ml, 1.0 ml or 0.5 ml of the freshly collected and filtered G-pseudotyped virus containing supernatant is added to the cells. The cells are incubated with the virus overnight at 37_C. On day 3 the medium is removed and 10 ml DMEM, 10% irradiated FBS with 800 μg/ml G418 (in the case of recombinant vectors carrying the *neo*R gene) is added to the plate. Only cells that have been transduced with the vector and contain the *neo* selectable marker will survive. A G418 resistant pool is generated over the period of 1-2 weeks. The pool is tested for

expression and then dilution cloned by removing the cells from the plate, counting the cell suspension, diluting the cell suspension down to 10 cells/ml and adding 0.1 ml to each well (1 cell/well) of a 96-well plate. Cells are incubated for 2 weeks at 37_C, 10% CO_2. Twenty-four clones are selected and expanded up to 24-well plates, then 6-well plates, and finally 10 cm plates, at which time the clones are assayed for expression and the supernatant are collected and assayed for viral titer as described above.

D.   Construction of Alphavirus Packaging Cell Lines

1.   Selection of Parent Cell Lines for Alphavirus Packaging Cell Line Development

a.   Persistently or Chronically Infectable Cells

An important criteria in selecting potential parent cell lines for the creation of alphavirus packaging cell lines is the choice of cell lines that exhibit little or no cytopathological effects prior to the appropriate production of alphavirus vector particles. This criteria is essential for the development of an alphavirus vector producer cell line which can be propagated for long periods of time and used as a stable source of vector. It is known that alphavirus infection of most mammalian cells results in cytopathology and lysis of the cell. However, the derivation of packaging cells from various insect cell lines may circumvent this problem. For example, insect cell lines, may be derived from *Aedes albopictus, Aedes aegypti, Spodoptera frugiperda*, and *Drosophila melanogaster* cells, may be utilized to construct alphavirus packaging cell lines. For example, within one embodiment, alphavirus packaging cell lines are provided using an insect parent cell line, such as from *Aedes albopictus* cell, containing a stably transfected expression vector cassette which allows for expression of alphavirus structural proteins under the control of inducible or non-inducible promoters active in these cell types, and co-expressing a selectable marker.

b.   Modification of Cells to Decrease Susceptibility to Alphavirus Expression: Suppression of Apoptosis and Cytopathology

Packaging cell lines may also be modified by overexpressing the *bcl*-2 gene product in potential parent cell lines, such as canine D-17 and CF2 cells; human HT1080 (ATCC #CCL121) and 293 cells; quail QT-6 cells; BHK-21 cells; mouse neuroblastoma N18 cells; and rat prostatic adenocarcinoma AT-3 cells. The conversion of these cells to a

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persistently infectable state allows for their use as alphavirus packaging and producer cell lines, similar to those of recombinant retroviral vector producer lines.

In order to construct such packaging cells, a *bcl*-2 expression vector is constructed by using standard recombinant DNA techniques in order to insert the 910 bp Eco RI cDNA fragment derived from the plasmid pB4 (Reed, *et. al.*, *Nature 336:259*, 1988) into any commercially available expression vector containing a constitutive promoter and encoding a selectable marker, for example, pCDNA3 (Invitrogen, San Diego, CA). Careful consideration must be taken to avoid any type of homology between alphavirus nucleic acid sequences and other transduced vectors. This precaution should be taken in order to prevent recombination events which may lead to undesirable packaging of selectable markers or the *bcl*-2 oncogene in recombinant Sindbis particles. This is an important point, since the alphavirus vector system described herein is designed for use as a biological therapeutic. Once the *bcl*-2 expression vector is constructed, the parent cell line (*i.e.*, BHK-21 cells) is transfected using any standard technique and selected after 24 hours using the appropriate marker. Resistant colonies are pooled, followed by dilution cloning, and then individual clones are propagated and screened for *bcl*-2 expression. Once expression is verified, persistent Sindbis infection is tested, followed by its use as a parent cell line for alphavirus packaging cell line development.

Other gene products in addition to the *bcl*-2 oncogene which suppress apoptosis may likewise be expressed in an alphavirus packaging or producer cell line. Three viral genes which are particularly preferred include: the adenovirus E1B gene encoding the 19-kD protein (Rao *et al.*, *PNAS 89*:7742-7746, 1992), the herpes simplex virus type 1 ₁34.5 gene (Chou and Roizman, *PNAS 89*:3266-3270, 1992), and the AcMNPV baculovirus p35 gene (Clem *et al.*, *Science 254*:1388-1390, 1991). These individual genes may be inserted into any commercially available plasmid expression vectors under the control of appropriate constitutive eukaryotic transcriptional promoters, and also containing a selectable marker using standard techniques. The expression vector constructs are subsequently transfected into cell lines as described above, and the appropriate selection is applied. Selection for stable integration of these genes and constitutive expression their products should allow for more extended vector production in cell lines found to be susceptible to alphavirus-induced apoptotic events. In addition, it is feasible that each gene product inhibits apoptosis by its own unique mechanism. Therefore, the genes may also be introduced into packaging or producer cell lines in various combinations in order to obtain a stronger suppressive effect. Finally, other gene products having similar effects on apoptosis can also be readily incorporated into packaging cell lines as they are identified.

In the derivation of alphavirus vector packaging and producer cell lines, many approaches can be employed to control the expression of viral genes such that producer cell

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lines stably transformed with both vector and vector packaging cassettes can be derived. These approaches include inducible and/or cellular differentiation sensitive promoters, antisense structural genes, heterologous control systems, and use of mosquito or other cells in which viral persistent infections can be established. Regardless of the final configuration

5   for the alphavirus vector producer cell line, the ability to establish persistent infection, or at least delay cell death as a result of viral gene expression, may be enhanced by inhibiting apoptosis. For example, the DNA tumor viruses, including adenovirus, HPV, SV40, and mouse polyomavirus (Py), transform cells in part by binding to and inactivating the retinoblastoma (Rb) gene product p105 and its closely related gene product, p107, and

10   other gene products involved in the control of the cell cycle including cyclin A, $p33^{cdk2}$ and $p34^{cdc2}$. All of these viruses, except for Py, encode gene products which bind to and inactivate p53. Uniquely, Py encodes the middle T antigen (mT) which binds to and activates the membrane tyrosine kinase, *src*, and also phosphatidylinositol-3-kinase, which is required for the full transformation potential of this virus (Talmage *et al.*, *Cell 59*:55-65,

15   1989). The binding to and inactivation of the Rb and p53 recessive oncogene products prevents cells transformed by these DNA tumor viruses from entering the apoptotic pathway. It is known that p53 is able to halt the division of cells, in part by inhibiting the expression of proteins associated with cellular proliferation, including *c-fos*, *hsc-70*, and *bcl-2* (Miyashita *et al.*, *Cancer Research 54*:3131-3135,1994).

20   In order to extend the duration of alphavirus vector production, or to promote a persistently infectable state, packaging and producer cells are preferably transformed with viral genomic DNA from Py or SV40. In particular, SV40 and Py transformed cell lines are established and the kinetics and level of Sindbis production and cytopathology after viral infection determined. If apoptic events characteristic of Sindbis proliferation in

25   hamster cells are diminished, each prototype alphavirus packaging and producer cell line subsequently is transformed with Py or SV40 in order to increase the yield of packaged vector from these cells.

    c.   <u>Modification of Cells to Decrease Susceptibility to Alphavirus</u>
30   <u>Expression: Production of Activation-Dependent Vector Particles</u>

    The Sindbis E2 glycoprotein is synthesized as a precursor, PE2. This PE2 precursor, along with the second viral glycoprotein, E1, associate in the endoplasmic reticulum and are processed and transported to the infected cell membrane as a heterodimer

35   for virion incorporation. At some point during this processing, PE2 is cleaved into E3 and the mature virion glycoprotein E2. E3 corresponds to the 64 amino-terminal residues of PE2 and is lost during maturation. The larger cleavage product, E2, is associated with E1

and anchored in what becomes the viral envelope. Host cell protease(s) is responsible for processing of the PE2 precursor, cleaving at a site that immediately follows a highly conserved canonical four amino acid (aa) residue motif, basic-X-basic-basic aa. A mutant cell line derived from the CHO-K1 strain, designated RPE.40 (Watson *et al.*, *J. Virol* 5 *65*:2332-2339, 1991), is defective in the production of Sindbis virus strain AR339 through its inability to process the PE2 precursor into the E3 and mature E2 forms. The envelopes of Sindbis virions produced in the RPE.40 cell line therefore contain a PE2/E1 heterodimer. RPE.40 cells are at least 100-fold more resistant to Sindbis virus infection than the parental CHO-K1. The defective virions produced by the RPE.40 cell line can be converted into a 10 fully infectious form by treatment with trypsin.

In packaging and producer cell lines, any wild-type alphavirus that is produced by recombination between vector and structural protein gene RNAs will re-infect cells and be rapidly amplified, significantly contaminating and decreasing the titer of packaged vector preparations. Packaging and producer cells developed from the RPE.40 line are an 15 alternative to other cell lines permissive for alphavirus infection due to the inefficient amplification of any wild-type virus generated during vector production and packaging. Thus, vector preparations are not significantly contaminated with wild-type virus. Furthermore, the benefits of this system are extended to other packaging and producer cell lines by developing "knock-out" mutants in their analogous cellular protease(s), using 20 techniques known in the art.

        d.   Hopping Cell Line Development

        Alphavirus hopping cell lines are used transiently to produce infectious RNA vector 25 particles which have been pseudotyped for a different cellular receptor tropism. Once the hopping cell line produces vector particles, it is no longer required because only the infectious culture supernatants are needed to transduce the original alphavirus packaging cell lines discussed above. Therefore, the hopping cell line need not exhibit persistent infection by alphavirus in order to transiently produce vector particles. In this instance, the 30 parent cell line can be either an insect cell line that exhibits persistent infection, or a mammalian cell line which is likely to lyse within 24-72 hours after a productive alphavirus infection. The only criteria is that the cell lines are able to express either VSV-G protein, with or without the appropriate alphavirus structural proteins, or retroviral *gag-pol* and *env* protein without affecting cell growth prior to introduction of the alphavirus RNA vector. 35 Therefore, the alphavirus hopping cell line can be any of the aforementioned parent cell lines able to support either alphavirus or retroviral replication, without the additional cell modifications discussed previously, such as *bcl*-2 oncogene expression. The generation of

VSV-G pseudotyped alphavirus vector particles can be accomplished by at least three alternative approaches, each of which is described in detail in U.S.S.N. 08/348,472.

For the pseudotyping of alphavirus vectors in retroviral packaging cell lines, any cell line which expresses retroviral *gag-pol* and *env* sequences may be used to package
5 alphaviral vectors that have been engineered to contain a retroviral packaging sequence. The retrovirus Ψ packaging sequence is inserted between the inactivated junction region and a synthetic junction region tandem repeat, such that only genomic-length vector, and not subgenomic RNA, is packaged by the viral envelope proteins. Viral-based particles containing alphavirus vector RNA may be produced by transfecting *in vitro* transcribed
10 alphavirus vector RNA using procedures described previously. Supernatants with pseudotyped retroviral particles containing alphavirus RNA vectors are harvested 24 hours post-transfection, and these supernatants can then be used to transduce an alphavirus packaging cell line.

15   e.   Identification of Parent Cell Lines which Produce Alphavirus
                   Resistant to Inactivation by Human Complement

Successful intravenous administration of recombinant alphavirus particles requires that the vector be resistant to inactivation in serum. It is well known to those skilled in the
20 art that Sindbis grown on BHK-21 cells is sensitive to complement-mediated serum inactivation, in terms of effective virus titer. To identify parent cell lines which produce Sindbis particles resistant to inactivation by human complement, the level of serum inactivation of Sindbis virus grown on multiple cell types is tested. The cell types tested are derived from many species, including human, for example, 293 or HT1080 cells.

25   As a source of human complement, approximately 70 mls of blood are collected from patients into serum separating tubes (Becton Dickinson, Los Angeles, CA). The blood is allowed to clot for one half hour at room temperature. After clotting the serum is centrifuged at 2000 xg for 10 minutes at 4_C. The serum is collected and placed into a 15 ml conical tube (Corning, Corning, NY) and placed on ice. Approximately, 1.1 ml aliquots
30 of the serum are placed in 2 ml cryovials, frozen in a dry ice/ethanol bath and stored at - 70_C for subsequent serum inactivation assays. Complement inactivated controls are prepared by heat inactivation of control aliquots for 30 minutes at 56_C.

To test Sindbis for serum inactivation, two vials containing 1.1 ml of 100% non-heat inactivated human serum are used for various virus preparations. One vial of serum is
35 quick thawed at 37_C. The serum is then heated to 56_C for 30 minutes to heat inactivate complement present in the serum. Following inactivation the serum is placed on ice. The second vial is quick thawed at 37_C. After thawing the serum is placed on ice.

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Approximately 1.0 ml of the non-heat inactivated serum, culture medium only, and heat-inactivated serum are placed in separate 1.5 ml tubes (Fisher Scientific, Pittsburgh, PA) and mixed with 1x10^5 pfu of Sindbis virus and incubated at 37_C for 1 hour. After incubation the tubes are placed on ice.

5   To identify the parent cell line host from which an alphavirus is resistant to human serum inactivation, the non-heat inactivated serum, medium, and heat-inactivated serum virus preparations are titered by plaque assay on BHK-21 cells. Equivalent virus titers regardless of incubation with non-heat inactivated serum, medium, or heat-inactivated serum are indicative of parent cell line hosts which produce Sindbis virus is resistant to
10   human complement inactivation.

2.   Structural Protein Expression Constructs

        a.   Inducible and Constitutive Structural Protein Vector Expression
                Cassettes
15

The development of alphavirus packaging cell lines is dependent on the ability to synthesize high intracellular levels of the necessary structural proteins: capsid, pE2 and/or E2, and E1. Unfortunately, high level expression of these proteins, in particular, the envelope glycoproteins E2 and E1, may lead to concomitant cytopathology and eventual
20   cell death. Therefore, structural protein expression cassettes have been designed with inducible regulatory elements which control the levels of gene expression, in addition to others which maintain constitutive levels of expression.

In a first configuration, expression of the alphavirus structural proteins is under control of the RSV LTR, in conjunction with the inducible *lac* operon sequences. This is
25   achieved by insertion of alphavirus cDNA corresponding to the viral structural protein genes into the pOP13 and pOPRSV1 vectors (Stratagene, San Diego, CA). These vectors, used separately, are co-transfected with the p3'SS vector (Stratagene, San Diego, CA), which expresses the *lac* repressor protein. In the absence of inducer, for example, Isopropyl-B-D-thiogalactopyranoside (IPTG), the basal, or constitutive, level of expression
30   of a luciferase reporter gene under *lac* operon control has been reported to be 10-20 copies per cell. Addition of IPTG results in a conformational change in the repressor protein which results in decreased affinity of the *lac* repressor protein for *lac*-operator sequences, permitting high level expression of the heterologous gene. Induction levels in the presence of IPTG of 95-fold have been reported for heterologous genes contained in the pOP13
35   vector.

Specifically, the Sindbis structural protein gene (SP) cDNA is inserted into the pOP13 and pOPRSV1 vectors as follows. The SP coding region is amplified *in toto* with a primer pair whose 5' ends map, respectively, to the authentic AUG translational start and UGA translational stop sites, including the surrounding nucleotides corresponding to the

5 Kozak consensus sequence for efficient translational initiation at Sindbis nt 7,638. The forward primer is complementary to Sindbis nts 7,638 to 7,661, and the reverse primer is complementary to Sindbis nt 11,384 to 11,364. PCR amplification of Sindbis cDNA corresponding to the structural protein genes is accomplished by a standard three-temperature cycling protocol using the following oligonucleotide pair:

10

Forward primer (7638F):

(Sequence ID No.: 49)

5'-TATATGCGGCCGCACCACCACCATGAATAGAGGATTCTTTAACATGC-3'

15

Reverse primer (11384R):

(Sequence ID No.: 50)

5'-TATATGCGGCCGCTCATCTTCGTGTGCTAGTCAG-3'

20

In addition to their respective complementarities to the indicated Sindbis nucleotide sequence, a 5 nucleotide "buffer sequence" followed by the Not I recognition sequence is included in the 5' end of each primer. Following PCR amplification, the 3,763 bp fragment is purified in a 1% agarose gel, then subsequently digested with Not I. The resulting 3,749

25 bp fragment is then ligated separately into the pOP13 and pOPRSV1 vectors which have been digested with Not I and treated with calf intestine alkaline phosphatase. These expression vector cassettes, which contain the entire coding capacity of the Sindbis structural proteins, are designated pOP13-SINSP and pOPRSV1-SINSP, respectively.

Variations of the *lac* operon-Sindbis structural protein gene expression cassettes

30 also can be constructed using other viral, mammalian, or insect-based promoters. Using common molecular biology techniques known in the art, the *lac* operon and the RSV LTR promoter, or just the RSV LTR promoter, can be switched out of the Stratagene pOP13 and pOPRSV1 vectors and replaced by other promoter sequences, such as the cytomegalovirus major immediate promoter (pOPCMV-SINSP); the adenovirus major late

35 promoter (pOPAMLP-SINSP); the SV40 promoter (pOPSV-SINSP): or insect promoter sequences, which include the Drosophila metallothionein inducible promoter (pMET-SINSP), Drosophila actin 5C distal promoter (pOPA5C-SINSP), heat shock promoters

HSP65 or HSP70 (pHSP-SINSP), or the baculovirus polyhedrin promoter (pPHED-SINSP).

b.   Inducible Expression of Structural Proteins Via Alphavirus Vector

Because of potential cytotoxic effects from alphavirus structural protein expression, the establishment of inducible packaging cell lines which express even modest basal levels of these proteins may not always be preferred. Therefore, packaging cell line expression cassettes are constructed which contain regulatory elements for the high level induction of structural protein synthesis via nonstructural proteins supplied in *trans* by the alphavirus vector, but with no basal level of synthesis until appropriately stimulated.

In this configuration, a structural protein gene cassette is constructed whereby transcription of the structural protein genes occurs from an adjacent alphavirus junction region sequence. The primary features of such a cassette are: an RNA polymerase II promoter positioned immediately adjacent to alphavirus nucleotide 1 such that transcription initiation begins with authentic alphavirus nucleotide 1; the 5'-end alphavirus sequences required for transcriptase recognition; the alphavirus junction region sequence for expression of the structural protein gene mRNA; the alphavirus structural protein gene sequences; the 3'-end alphavirus sequences required for replication; and a transcription termination/polyadenylation sequence. Because of an upstream open-reading frame which ends prior to the AUG start site of the structural protein genes, expression of the alphavirus structural proteins can occur only after the synthesis of minus-strand RNA by vector-supplied nonstructural proteins, followed by the subsequent transcription of a structural protein gene mRNA from the junction region. Therefore, the inducibility of this system is dependent entirely on the presence of nonstructural proteins supplied by the alphavirus vector itself, introduced as either RNA transcribed *in vitro* or cDNA positioned downstream of an appropriate promoter element. In addition, the 5'- and 3'-end alphavirus sequences allow for this RNA transcript of the structural protein gene cassette to be amplified by the same vector-supplied nonstructural proteins (*see* Figure 16).

Specifically, the construction of a positive-sense, vector-inducible Sindbis packaging cassette is accomplished as follows. Briefly, the pVGELVIS vector described previously is digested with the enzyme Bsp EI to remove nucleotides 422 to 7054, including most of the nonstructural gene coding sequences, and the remaining 9925 bp fragment is purified in a 0.8% agarose gel, and subsequently re-ligated to itself to generate the construct designated pLTR/SindlBspE (Figure 16). This deletion leaves the 5'-end authentic translation start codon at nts 60-62 intact and creates in-frame downstream UAA and UGA stop codons at nts 7,130 to 7,132 and 7,190 to 7,192, respectively, thus

preventing translation of the downstream structural protein gene open-reading frame. The pLTR/SindlBspE packaging cassette construct is subsequently transfected into BHK-21 cells and transfectants are selected using G418 at 400 µg/ml and cloned by limiting dilution. After expansion of the transfected clonal lines, screening for packaging activity is

5   performed by transfection of Sindbis-luciferase (SIN-luc) vector RNA as described previously. The data shown in Figure 17 demonstrate that transfection of SIN-luc vector RNA into several of these clonal LTR/SindlBspE packaging cells results in the production of infectious Sindbis particles containing the SIN-luc RNA, as the recovered supernatants are shown to transfer SIN-luc vector RNA to fresh monolayers of BHK-21 cells.

10   A wide variety of variations of these packaging cassette constructions can be made given the disclosure provided herein, including, for example, the substitution of other RNA polymerase promoters for the current MuLV LTR, the addition of one or more nucleotides between the RNA polymerase promoter and the first Sindbis nucleotide, the substitution of other ribozyme processing sequences, or the substitution of a non-Sindbis-encoded open

15   reading frame upstream of the structural protein gene sequences, which may or may not retain the 5'-end Sindbis sequences required for transcriptase recognition. Furthermore, these constructs can be transfected into other cell lines, as discussed previously.

           In another vector-inducible packaging configuration, expression cassettes contain a cDNA copy of the alphavirus structural protein gene sequences flanked by their natural

20   junction and 3'-untranslated regions, and are inserted into an expression vector in an orientation such that primary transcription from the promoter produces antisense structural protein gene RNA molecules. Additionally, these constructs contain adjacent to the junction region alphavirus 5'-end sequences necessary for recognition by the viral transcriptase and a catalytic ribozyme sequence positioned immediately adjacent to

25   alphavirus nucleotide 1 of the 5'-end sequence. As such, this ribozyme cleaves the primary RNA transcript precisely after the first alphavirus nucleotide. In this antisense orientation, the structural protein genes cannot be translated, and are dependent entirely on the presence of alphavirus virus nonstructural proteins for transcription into positive-strand mRNA prior to their expression. These nonstructural proteins again are provided by the

30   alphavirus vector itself. In addition, because this configuration contains the precise alphavirus genome 5'- and 3'-end sequences, the structural protein gene transcripts undergo amplification by utilizing the same nonstructural proteins provided by the alphavirus vector. Details of this construction are described in (U.S.S.N. 08/348,472).

           In addition, packaging cell lines also are generated which segregate the integration

35   and expression of the structural protein genes, allowing for their transcription as non-overlapping, independent RNA molecules, using the approaches described above. For example, the expression of capsid protein independently of glycoproteins E2 and E1, or

each of the three proteins independent of each other, eliminates the possibility of recombination with vector RNA and subsequent generation of contaminating wild-type virus. The construction of such "split gene" expression cassettes is detailed in (U.S.S.N. 08/348,472).

c.   Assembling the Components to Create the Alphavirus Packaging
      Cell Line

For example purposes, the BHK-21 cell line and replicon-inducible packaging expression cassette are used to demonstrate assembly of the components. However, other possible parent cell lines can be used to create alphavirus packaging cell lines and have been discussed previously. Briefly, BHK-21 cells are grown at 37_C in 5% CO_2 in DMEM, 2 mM L-glutamine, and 10% fetal bovine serum (optimal media). Approximately 5×10^5 BHK-21 cells, grown in a 35 mM petri dish, are transfected with 5 µg pLTR/SindIBspE using 5 µl of the Transfectam (Promega, Madison, WI) cationic lipid reagent in serum-free media conditions, as suggested by the supplier. However, any method of transfection can be rapidly substituted, i.e., electroporation, calcium phosphate precipitation, or by using any of the readily available cationic liposome formulations and procedures commonly known in the art. At 24 hours post-transfection, the cells are trypsinized and reseeded in 100 mm dishes in 10 ml of optimal media, as described above, supplemented with 400 µg/ml of G418 and selected over a period of 5 to 7 days. Colonies displaying resistance to the G418 drug are then pooled, dilution cloned, and propagated. Individual clones are screened for high levels of Sindbis structural protein expression and functional packaging after transfection with Sindbis-luciferase vector RNA transcribed in vitro from SacI linearized plasmid pKSSINBV-luc (see Example 1C(iv)). Specifically, clonally-derived pLTR/SindIBspE transfected BHK-21 cells (referred to as LTR/SindIBspE or BK-Bsp cells) grown in 60 mm petri dishes are transfected with 2 µg of Sindbis-luciferase vector RNA and overlayed with 3 ml of optimal media (see above). At 20 hours post-transfection, the supernatants are removed and clarified by centrifugation for 30 minutes at 3,000 rpm in a Sorvall RT6000B tabletop centrifuge. In addition, the transfected cell monolayer is lysed in reporter lysis buffer (Promega, Madison, WI) as described by the manufacturer and assayed for luciferase expression as described previously.

The transfer of luciferase activity (and thus functional packaging) is tested by using 1.0 ml of the above supernatants to infect fresh monolayers of BHK-21 cells in 60 mm dishes. At 20 hours post-infection, the cell monolayers are lysed as described above and tested for luciferase expression. As shown in Figure 17, three clones (#13, 18, and 40) produce packaged Sindbis-luciferase vector. In addition, transfected clone #18 cells are

tested for increased vector packaging over a timecourse following transfection. Supernatants from transfected clone #18 cells are harvested at 20, 45, and 70 hours post-transfection, as described above, and used to infect fresh monolayers of BHK-21 cells. Figure 18 shows that Sindbis-luciferase vector packaging increases significantly at 45 hours

5   post-transfection. Expression also can be tested by Western blot analysis using polyclonal rabbit anti-Sindbis antibodies.

3.   **Inducible Vector and Structural Protein Expression for Alphavirus Producer Cell Lines**

10   a.   **Use of Viral Promoters**

Another approach to developing an alphavirus vector producer cell line is to generate alphavirus vector producer lines from mosquito cells, where viral persistence often results after infection. However, the titer of infectious virus produced in persistently

15   infected mosquito cells is only about 1.0 x 10^4 pfu/ml, at least five orders of magnitude less than that observed after lytic infection of BHK-21 cells by Sindbis.

Several strategies are described for inducible alphavirus vector producer cell lines containing both vector and viral structural gene cassettes such that productive cytolytic infection occurs only after the correct stimulus. Because these approaches operate on a

20   "feed forward" level, "leakiness" in the regulatory control of the system may result in initiation of the alphavirus replication cycle and probable cell death. Therefore, tightly regulated control mechanisms are necessary for such a system.

The hallmark of development is the differentiation state-dependent pattern of gene expression. Briefly, gene expression patterns differ widely between undifferentiated and

25   terminally differentiated states. Thus, a cell whose differentiation state can be controlled is likely an ideal host in which to derive an alphavirus vector producer cell line. In such a configuration, the expression vector cassette and, in some instances, structural components are coupled to terminal differentiation state-inducible promoters, according to the strategy described for ELVIS, and used to transform stably an undifferentiated host cell. Terminal

30   differentiation of the host producer cell after induction with the appropriate stimuli coincidentally results in induction of the alphavirus replication cycle and production of packaged vector. Other strategies described herein, including antisense structural genes and heterologous viral expression systems, are readily coupled with cellular differentiation state-dependent promoters described below.

35   In this approach, four examples are described using either a viral or cellular promoter active in only terminally differentiated cells.

It has been shown that mouse Py, SV40, and MoMLV are all able to infect and enter undifferentiated mouse embryonal carcinoma (EC) cells, but the expression of their genes (and heterologous genes) and establishment of productive infection is blocked (Swartzendruber and Lehman, *J. Cell. Physiol.* 85:179-188, 1975; Peries *et al.*, *J. Natl.*
5   *Cancer Inst.* 59:463-465, 1977). These viral growth properties also have been demonstrated in two cell lines, PCC4 and F9, which are derived from the malignant stem cells of mouse teratorcarcinomas. The block to viral propagation occurs at the level of transcription and replication, and maps to the enhancers contained within the viral non-coding control regions (Linney *et al.*, *Nature* 308:470-472, 1984; Fujimura *et al.*, *Cell*
10   23:809-814, 1981; Katinka and Yaniv, *Cell* 20:393-399, 1980). When MoMLV infects undifferentiated EC cells, the viral DNA integrates into the genome. However, as stated above, expression of viral or heterologous genes is blocked. This block of viral expression is released upon terminal differentiation of EC cells by addition of retinoic acid to the growth medium.

15   To test the RNA expression properties of the pVGELVIS construct in EC cells, plasmid DNA is complexed with Lipofectamine™ according to the conditions suggested by the supplier (*ca.*, 5 g DNA/8 g lipid reagent) and added to 35 mm wells containing undifferentiated PCC4 or F9 cells (Fujimura *et al.*, 1981, *Cell* 23:809-814) at approximately 75% confluency. The development of cpe and the level of Sindbis
20   productive infection, quantitated by plaque assay of media supernatant, is determined at regular intervals over 5 days in undifferentiated and differentiated transfected PCC4 or F9 cells. Differentiation of F9 and PCC4 cells is accomplished by addition of retinoic acid (Sigma Chemical Co., St. Louis, MO), to a final concentration of 1 M.

It has been proposed that the hierarchy of relative expression of heterologous genes
25   observed in undifferentiated EC cells infected with MoMLV vectors may be in part insertion dependent (Linney *et al.*, 1987, *J. Virol.* 61:3248-3253). Thus, undifferentiated EC cells transfected with pVGELVIS may likely produce different results, in terms of transcription of the Sindbis genomic cDNA and, in turn, of initiation of the viral life cycle. In this event, following G418 selection of pVGELVIS transfected undifferentiated EC cells,
30   remaining cells are cloned and expanded. The cell clones are then tested for the production of Sindbis virus after differentiation by addition of retinoic acid.

To isolate vector packaging cell lines whose production of structural proteins in the presence of Sindbis nonstructural proteins is cell differentiation state dependent, undifferentiated F9 or PCC4 cells are transfected with pLTR/SINdlBspE and G418
35   selected, as described above. Differentiation state-sensitive clones are then selected by infection at high multiplicity with packaged pSIN-BV-luc vector. Clones which are resistant to cell lysis or do not produce packaged pSIN-BV-luc vector particles, are

candidate vector packaging clones. These candidate clones are tested for SIN-luc vector particle production following terminal differentiation with retinoic acid, as described.

The murine wild type Py is unable to replicate in the teratocarcinoma cell lines PCC4 or F9. This block of replication in undifferentiated cells occurs at the level of
5   transcription of early region (*i.e.*, T antigen) genes, and is released by induction of terminal differentiation with vitamin A. Py mutants which are able to establish productive infection in undifferentiated PCC4 and F9 cells map to the viral enhancer region. The genesis of an embryonic tissue specific transcriptional enhancer element has resulted in these mutants. In order to exploit this property of inhibition of Py replication in undifferentiated
10 teratocarcinoma cell lines, the viral regulatory non-coding region, including the enhancer, is coupled to the genomic cDNA of Sindbis virus according to the ELVIS strategy. The precise transcriptional start site of the Py early region has been determined (*see* Tooze, DNA Tumor Viruses, 2nd Ed. Cold Spring Harbor, NY, 1981). The PCC4 and F9 cell lines are stably transformed with the Py-Sindbis vectors. Sindbis productive infection
15 occurs after addition of retinoic acid to the culture medium and induction of terminal differentiation. The construction of differentiation state-controlled alphavirus vectors is detailed in (U.S.S.N. 08/348,472).

        b.   Use of Cellular Promoters
20

The third example of this strategy uses the β-globin locus control region. The β-globin multigene cluster contains five developmentally regulated genes. In the early stages of human development, the embryonic yolk sac is the hematopoietic tissue and expresses the ε-globin gene. This is followed by a switch to the γ-globin gene in the fetal liver and the
25 δ- and β-globin genes in adult bone marrow (Collins and Weissman, 1984, *Prog. Nucleic. Acid Res. Mol. Biol. 31*:315).

At least two mouse erythroleukemia lines, MEL and Friend, serve as models for terminal differentiation dependent expression of β-globin. Expression of β-globin is observed in these lines only after induction of terminal differentiation by addition of 2%
30 DMSO to the growth medium.

The entire β-globin locus is regulated by the locus control region (LCR). Within the LCR is the dominant control region (DCR) residing within the DNase I hypersensitive region, which is 5' of the coding region. The DCR contains five DNase I hypersensitive (HS1- HS5) sites. The DCR directs high level site of integration independent, copy number
35 dependent expression on a linked human β-globin gene in transgenic mice and stably transfected mouse erythroleukemia (MEL) cells (Grosveld *et al., CSHSQB 58*:7-12, 1993).

In a recent study (Ellis *et al.*, *EMBO 12*:127-134, 1993), concatamers of a synthetic core coinciding to sequences within HS2 were shown to function as a locus control region.

5   In order to accomplish the differentiation state dependent expression of alphavirus vectors, the viral genomic cDNA is juxtaposed with a promoter containing a tandem synthetic core corresponding to the LCR HS2 site. Alternatively, the desired alphavirus vector construct can be inserted downstream of the LCR in the endogenous β-globin gene by homologous recombination. In such a strategy, the β-globin transcription initiation site after terminal differentiation would be first determined, in order that the alphavirus vector could be placed precisely at the start site.

10   Initiation of a lytic viral life cycle is controlled by the differentiation state of the host cell is applicable to other systems, where the control of viral induced cytopathology is desired.

       Yet another approach to regulating alphavirus gene expression through a differentiation state sensitive promoter is the use of the retinoic acid receptor a (RARA)

15   and acute promyelomonocytic leukemia cells (APL). APL cells are clonal myeloid precursors characterized by high growth rate and differentiation arrest. A non-random chromosomal translocation breakpoint, t(15;17)(q22;21), occurs in almost all patients with APL. The RARA gene has been localized to chromosome 17q21. Analysis of APL mRNA from patients has shown that most APL breakpoints occur within the second intron of the

20   RARA gene and result in abnormal fusion transcripts. Co-transfection assays with RARA and PML-RARA fusion cDNAs have demonstrated that the resulting fusion proteins can antagonize wild-type RARA in the presence of retinoic acid. These studies implicate PML-RARA fusion protein in the molecular pathogenesis of APL. Importantly, a significant number of patients achieve complete remission after all-trans retinoic acid treatment

25   (ATRA). High concentration of ATRA may overcome the RARA deficiency leading to high levels of RA in the nucleus. Differentiation of the APL cells can then be achieved through activation of RARA responsive genes. RA can induce differentiation of a number of cell lines, including the human leukemia line HL-60.

       The retinoic acid receptor is a member of a nuclear receptor superfamily that

30   includes the thyroid and steroid hormone receptors. Four different forms of the human RAR have been identified, and the corresponding cDNAs cloned and characterized. In order to accomplish the differentiation state dependent expression of Sindbis vectors, viral genomic cDNA is juxtaposed with the RARA DNA binding site, creating ELVIS-RARASIN. As with the strategy proposed for ELVIS-PySIN expression in

35   undifferentiated EC cells, differentiation sensitive ELVIS-RARASIN expressing cells are isolated.

c.   Insertion of Vector Constructs into Differentiation State Controlled
        Inducible Promoters

        Generation of clones, whose expression of heterologous genes from Sindbis vectors
5   positioned in the ELVIS configuration is differentiation state dependent is accomplished as
     described above for the pVGELVIS and pLTR/SindIBspE plasmids. Generation of clones
     whose production of vector particles is differentiation state dependent is accomplished by
     transfecting the isolated differentiation dependent vector packaging clones described above
     with ELVIS heterologous gene expression vectors. Clones having the desired phenotype
10   or vector production after retinoic acid induced differentiation are isolated as described
     above.

E.   Creation of CEA RNA Dependent Sindbis Vector Producer Cell Lines

15   Unlike the previous examples of creating producer cell lines, it may be that only a
     single round of gene transfer into the packaging cell line is possible by vector transfection.
     Since these vectors will be disabled and prevented in the synthesis of full genomic vectors,
     re-infection of a fresh layer of Sindbis packaging cell lines will end in an aborted infection
     since these vectors are now dependent on the presence of the CEA RNA to become active.
20   Higher titers may be achieved by dilution cloning transfected producer cell lines using the
     RT-PCR technique.

Example 3

A.   The Extended S^+L^- Assay

        The extended S^+L^- assay determines whether replication competent, infectious virus
30   (RCR) is present in the supernatant of the cell line of interest. The assay is based on the
     empirical observation that infectious retroviruses generate foci on the indicator cell line
     $MiCl_1$ (ATCC No. CCL 64.1). The $MiCl_1$ cell line is derived from the Mv1Lu mink cell
     line (ATCC No. CCL 64) by transduction with murine sarcoma virus (MSV). It is a non-
     producer, non-transformed, revertant clone containing a replication defective murine
35   sarcoma provirus, S^+, but not a replication competent murine leukemia provirus, L^-.
     Infection of $MiCl_1$ cells with replication competent retrovirus "activates" the MSV genome
     to trigger "transformation" which results in foci formation.

Supernatant is removed from the cell line to be tested for presence of replication competent retrovirus and passed through a 0.45 µ filter to remove any cells. On day 1, Mv1Lu cells are seeded at 1.0×10^5 cells per well (one well per sample to be tested) of a 6 well plate in 2 ml DMEM, 10% FBS and 8 µg/ml polybrene. Mv1Lu cells are plated in the same manner for positive and negative controls on separate 6 well plates. The cells are incubated overnight at 37_C, 10% CO_2. On day 2, 1.0 ml of test supernatant is added to the Mv1Lu cells. The negative control plates are incubated with 1.0 ml of media. The positive control consists of three dilutions (200 focus forming units (ffu), 20 ffu and 2 ffu each in 1.0 ml media) of MA virus (refered to as pAM in Miller *et al.*, *Molec. and Cell Biol.* 5:431, 1985) which is added to the cells in the positive control wells. The cells are incubated overnight. On day 3, the media is aspirated and 3.0 ml of fresh DMEM and 10% FBS is added to the cells. The cells are allowed to grow to confluency and are split 1:10 on day 6 and day 10, amplifying any replication competent retrovirus. On day 13, the media on the Mv1Lu cells is aspirated and 2.0 ml DMEM and 10% FBS is added to the cells. In addition, the MiCl₁ cells are seeded at 1.0×10^5 cells per well in 2.0 ml DMEM, 10% FBS and 8 µg/ml polybrene. On day 14, the supernatant from the Mv1Lu cells is transferred to the corresponding well of the MiCl₁ cells and incubated overnight at 37_C, 10% CO_2. On day 15, the media is aspirated and 3.0 ml of fresh DMEM and 10% FBS is added to the cells. On day 21, the cells are examined for focus formation (appearing as clustered, refractile cells that overgrow the monolayer and remain attached) on the monolayer of cells. The test article is determined to be contaminated with replication competent retrovirus if foci appear on the MiCl₁ cells. Using these procedures, it can be shown that the recombinant viral vector producer cell lines are not contaminated with replication competent retroviruses.

B.   Co-Cultivation of Producer Lines and MdH Marker Rescue Assay

As an alternate method to test for the presence of RCR in a vector-producing cell line, producer cells are cocultivated with an equivalent number of *Mus dunni* (NIH NIAID Bethesda, MD) cells. Small scale cocultivations are performed by mixing of 5.0×10^5 *Mus dunni* cells with 5.0×10^5 producer cells and seeding the mixture into 10 cm plates (10 ml standard culture media/plate, 4 µg/ml polybrene) at day 0. Every 3 to 4 days the cultures are split at a 1:10 ratio and 5.0×10^5 *Mus dunni* cells are added to each culture plate to effectively dilute out the producer cell line and provide maximum amplifcation of RCR. On day 14, culture supernatant is harvested, passed through a 0.45 µ cellulose-acetate filter, and tested in the MdH marker rescue assay. Large scale co-cultivations are performed by seeding a mixture of 1.0×10^8 *Mus dunni* cells and 1.0×10^8 producer cells into a total of

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twenty T-150 flasks (30 ml standard culture media/flask, 4 µg/ml polybrene). Cultures are split at a ratio of 1:10 on days 3, 6, and 13 and at a ratio of 1:20 on day 9. On day 15, the final supernatant is harvested, filtered and a portion of each is tested in the MdH marker rescue assay.

5   The MdH marker rescue cell line is cloned from a pool of *Mus dunni* cells transduced with LHL, a retroviral vector encoding the hygromycin B resistance gene (Palmer *et al.*, *PNAS 84*: 1055-1059, 1987). The retroviral vector can be rescued from MdH cells upon infection of the cells with RCR. One ml of test sample is added to a well of a 6-well plate containing 1.0×10^5 MdH cells in 2 ml standard culture medium (DMEM

10   with 10% FBS, 1% 200 mM L-glutamine, 1% non-essential amino acids) containing 4 µg/ml polybrene. Media is replaced after 24 hours with standard culture medium without polybrene. Two days later, the entire volume of MdH culture supernatant is passed through a 0.45 µ cellulose-acetate filter and transferred to a well of a 6-well plate containing 5.0×10^4 *Mus dunni* target cells in 2 ml standard culture medium containing

15   polybrene. After 24 hours, supernatant is replaced with standard culture media containing 250 µg/ml of hygromycin B and subsequently replaced on days 2 and 5 with media containing 200 µg/ml of hygromycin B. Colonies resistant to hygromycin B appear and are visualized on day 9 post-selection by staining with 0.2% Coomassie blue.

20   Example 4

                          Determination of Protein Expression

A.   Western Blotting

25
       i.   Preparation of RIPA-Lysates

       Cells transduced with the recombinant vectors of this invention are harvested with trypsin/EDTA and washed twice with cold PBS. The cells are lysed with 50 to 400 ml

30   RIPA buffer (10 mM Tris-HCl pH 7.0; 1% (v/v) NP40; 0.1% (w/v) SDS; and 150 mM NaCl) and incubated for 15 minutes at room temperature. The lysate is centrifuged at full speed in an Eppendorf centrifuge for 5 minutes. The supernatant is removed and stored at -20_C. A Bradford protein assay is performed to determine the total protein concentration.

35
       ii.   SDS-Page

       A sample of RIPA lysate containing a total protein concentration of 20 mg and a commercial molecular weight (MW) marker (Amersham, Chicago, IL) are mixed 1:1 with

2x sample buffer (4% (w/v) SDS, 50 mM Tris-HCl pH 7.0, 24% (v/v) glycerol, 0.1% (w/v) bromophenol blue and 0.05% β-mercaptoethanol) and heated to 65_C for 10 minutes. After heating the sample and MW marker are placed on ice. The slots of a precast 7.5% Tris HCl based minigel (BioRad, Hercules, CA) are rinsed with running buffer (3.0 gm
5 Tris-HCl pH 8.6, 1.0 gm SDS and 14.4 gm glycine to 1.0 L) and the sample and MW marker are loaded onto the gel. Approximately 70 to 120V is applied to the gel until the marker reaches the bottom of the gel.

10   iii.   <u>Transfer</u>

The protein bands are transferred from the gel to an Immobilon-P™ membrane (Millipore, Bedford, MA) by immersing the gel in CAPS buffer pH 11.0 with 5% (v/v) methanol for 5 minutes. The Hoefer HSI TTE transfer apparatus (Hoefer Scientific
15 Instruments, San Francisco, CA) is used to transfer proteins from the gel to the membrane. Approximately 70V is applied to the gel for 1.5 hours.

          iv.   <u>Immunodetection</u>
20

The Immobilon-P™ membrane is blocked for 30 minutes at room temperature with 0.5% BM blocking reagent (Boehringer Mannheim, Chicago, IL) containing 3% BSA, heated slowly in a microwave. The membrane is then probed with a primary mouse antibody that reacts specifically with the protein being detected at a 1:2000 dilution in BM
25 blocking solution containing a 3% BSA and incubated for 1 hour at room temperature. The membrane is washed three times with 40 ml of PBST (0.2% Tween-20 in PBS) for 10 min and a secondary goat anti-mouse, HRP-labeled antibody (Jackson, Bar Harbor, MA) at a 1:20,000 dilution in 3% BSA solution (Sigma, St Louis, MO). The membrane is then washed three times with 40 ml of PBST for 10 minutes. After washing, the membrane is
30 submersed in ECL developing solution (Amersham, Chicago, IL) for 1 minute and then wrapped in plastic wrap and exposed to Hyperfilm (Amersham, Chicago, IL) for 5 seconds. The film is then developed and the protein bands are analyzed.

35   B.   <u>Indirect Immunofluorescence Staining and FACS Analysis</u>

The indirect immunofluorescence staining and FACS analysis method of Amlot, *et al.* (Lymphocytes: A Practical Approach, GGB Klaus (ed.), IRL Press (1987), pp 77-72) is

used to detect cell surface expression of heterologous protein antigens from recombinant vector transduced cells. Fifty microliters of primary antibody stock, 62 µg/ml of antibody that specifically react with the protein to be detectedin FACS buffer PBSA containing 0.2% BSA and 0.2% NaN$_3$ are added to each well in the first column of a 96 well U-shaped
5   microtiter plate. The antibody in this column is then serially diluted to the wells of the remaining columns. Approximately 50 µl of target cell suspension, 2.0 x 10^6 to 4.0 x 10^6 cells/ml, in FACS buffer are placed in each well (the antibody final concentrations are 31, 10, 3.1, 0.31, and 0.1 µg/ml) of the microtiter plate and incubated for 15 minutes at room temperature. Following incubation, 100 µl of FACS buffer is added to each well and the
10   microtiter plate is centrifuged at 2000 rpm for 1 minute. The supernatant is removed and 200 µl of fresh FACS buffer is added to each well. This process is repeated 3 additional times. After the final wash, the secondary antibody solution containing 85% FACS buffer, 10% normal mouse serum, and 5% FITC labeled anti-mouse antibody is added. The microtiter plate is washed four times with fresh FACS buffer. After the final wash, 150 µl
15   from each well is transferred to a FACS tube (Falcon 2052 tubes) and 100 µl of FACS buffer is added. The fluorescent signal is detected by a FACScan counter.

C.   **Simple Indirect Immuno-peroxidase Staining on Frozen Sections**

20   Frozen tissue sections are dried at room temperature for 30 minutes. The dried tissue sections are fixed with anhydrous acetone for 15 minutes at room temperature and allowed to air dry. The sections are rehydrated with PBS for several minutes. Tissue sections are then incubated in a humid chamber for 45 to 120 minutes at room temperature with 50 µl of primary antibody that specifically reacts with the protein to be detected
25   (Marek, *et al.*, *Cancer 67*:1377, 1991). The tissue sections are then washed with PBS and allowed to soak in PBS for several minutes. Following soaking, the sections are incubated with 50 µl of HRP conjugated anti-mouse antibody or HRP-conjugated strept-avidin (DAKO, Carpenteria, CA) for 45 to 120 minutes at room temperature. The tissue sections are then washed with PBS and allowed to soak in PBS for several minutes. Following
30   soaking, the sections are incubated with 60 to 100 µl substrate solution containing 0.01% H$_2$O$_2$ and 3,3 diaminodenzidine (DAB) for 8 minutes or for 20 minutes with substrate solution containing 100 µl of α-ethyl carbazole (60 mg of 3-amino-9-ethylcarbazole in 25 ml DMF) in 1 ml of acetate buffer (0.68 g sodium acetate in H$_2$O pH 5.2 adjusted to a volume of 250 mls) and 0.01% H$_2$O$_2$. The tissue sections are washed with water for 2-5
35   minutes and then stained with haemotoxylin for 15 seconds. The tissue sections are then washed with water for 10 seconds and mounted in a Crystal Mount™ (Biomeda, Alameda, CA) according to manufacturer's instructions.

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D.   Expression of Luciferase in Transfected and Infected BHK-21 Cells

5   In order to test the functionality of the Sindbis Basic Vector, the expression of
luciferase in cells transfected with RNA transcribed *in vitro* from Sac I-linearized
pKSSINBV-luc is tested. In addition, a complementary packaging vector which is deleted
of most of the nonstructural gene region is constructed by digestion of pVGSP6GENrep
with Bsp EI and re-ligation under dilute conditions. This construction, designated
10   pVGSP6GENdlBsp, lacks nonstructural gene sequences between bases 422-7,054.
Transcription *in vitro* of Xba I-linearized pVGSP6GENdlBsp and Sac I linearized
pKSSINBV-luc is performed as described previously. Transfections and co-transfections
are performed by complexing *in vitro* transcription products with Lipofectin™ and applying
to BHK-21 cells. The expression of luciferase in transfected cells is tested 18 hours after
15   transfection. Additionally, 1.0 ml of the transfection supernatant is used to infect a
confluent monolayer of BHK-21 cells and the expression of luciferase is tested at 24 hours
post-infection.

          The results of this experiment, shown in Figure 19, demonstrate clearly abundant
reporter gene expression follows transfection of BHK-21 cells with *in vitro* transcribed
20   RNA from pKSSINBV-luc, and transfer (*e.g.*, packaging) of the expression activity when
cells are co-transfected with *in vitro* transcribed RNA from pVGSP6GENdlBsp.

          Additonal modifications to the junction region promoter which increase, decrease,
or inactivate activity, or result in the insertion of tandemly arranged copies of the junction
region, are readily accomplished based on (U.S.S.N. 08/348,472).

25   To detect intercellular markers, such as HSV TK, a FACS analysis is performed.
Briefly, HT1080 cells are transiently transduced with varying dilutions of the recombinant
viral vector carrying the HSV TK gene. The samples are centrifuges and the cells are
resuspended in 2.0 ml of PBS that does not contain Ca^{+2} or Mg^{+2} (CMF.PBS).
Approximately 0.2 ml of 37% formaldehyde solution is added while the sample is gently
30   shaken. The sample is then incubated for 20 minutes at room temperature with gentle
agitation. Following incubation, the sample is washed three times with CMF.PBS. The
cells are then suspended in 2.0 ml of 50 mM NH_4Cl in CMF.PBS and incubated for 20
minutes at RT with gentle agitation. Following incubation, the sample is washed twice with
CMF.PBS. The sample is then washed again with CMF.PBS containing 1% BVSA
35   (Fraction V, Sigma, St. Louis, MO) and 1% saponin (CMF.PBS/BSA/Sap).
Approximately 100 µl of primary antibody (Repligen, Cambridge, MA; 1C1 0.5 µg/sample)
is added to this suspension and the mixture is incubated for 1.0 hour at RT with gentle

agitation. The mixture is then centrifuged and the pellet is washed three times with CMF.PBS/BSA/Sap. Approximately 100 µl of dilute secondary antibody (Cappel, Durham, NC; rabbit anti-mouse IgG-FITC 1:1000) resuspended in CMF.PBS/BSA/Sap and the mixture is incubated for 30 minutes at RT with gentle agitation. Following
5 incubation, the pellet is washed three times with CMF.PBS/BSA/Sap. The sample is resuspended in 0.5 ml of CMF.PBS/BSA/Sap containing 25 µg of Propidium Iodide and analyzed by FACS.

Example 5

10

Determination of Activity of the Expressed Protein

A.   Determination of Tissue Factor Activity   ·

15   Cells are removed from plates by suspension in 1 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl. The absorbance at 550 nM is adjusted to 0.750 in order to adjust for differential cell density on the plates. The cells are sonicated for 1 minute and Triton X-100 is added to a final concentration of 0.1 percent. The samples are rotated at room temperature for 90 minutes and cellular debris removed by centrifugation
20 at 10,000 x g. Detergent solubilized extracts are relipidated by diluting 2 µl of the sample into 0.8 ml 0.05 M Tris-HCl, pH 7.5, containing 0.1 M NaCl, 0.1 percent bovine serum albumin (TBS buffer). Fifty µl of a 5 mg/ml solution of phosphotidylcholine (lecithin) in 0.25% deoxycholic acid and 25 µl of $CdCl_2$ are added and the solution incubated for 30 minutes at 37_C.

25   The chromogenic assay for tissue factor activity is based upon the activation of factor X in the presence of tissue factor and factor VII. The amount of factor X_a formed is then assessed by the factor X_a catalyzed cleavage of the chromogenic substrate S2222. Three µl of a 0.4 mg/ml solution of human factor X (Enzyme Research Laboratories, South Bend, IN) and 2 µl of a 200 U/ml solution of human factor VII (Sigma Chemical Company,
30 St. Louis, MO) are added to 100 µl of 0.025 M CaCl and 46 µl of TBS. The sample to be assayed is added and the reaction mixture incubated for 5 minutes at 37_C. The chromogenic substrate S2222 (Helena Laboratories, Beaumont, TX) is added (50 µl of a 2 mg/ml solution) and the reaction allowed to continue for 10 minutes. The reaction is terminated by the addition of 100 µl of glacial acetic acid and the absorbance at 405 nm is
35 measured. An assay control consisted of the addition of a relipidation mixture to which TBS buffer is added instread of a tissue factor containing sample.

For the assay of COS-7 transfected cells, 2 µl of cell extract is added to the reaction mixture. For reference, a standard curve was constructed using rabbit brain thromboplastin (Sigma Chemical Co., St. Louis, MO) reconstituted according to the manufacturer's instructions. The thromboplastin solution is diluted 1:30 and the indicated volumes are

5   added to the chromogenic assay (Fisher, et al., *Thrombosis Research* 48:89, 1987).

B.   Determination of Anti-Angiogenic Activity

Anti-angiogenic activity of various polypeptides useful in accordance with certain

10   aspects of this invention may be assayed on the chorioallanoic membrane (CAM) as described by Takigawa, et al., (*Biochem. Int.* 14:357, 1987). Briefly, B16 melanoma cells are inoculated subcutaneously into the loins of C57BL/6N mice. When the tumors reach approximately 1 cm in diameter, they are excised, cut into pieces of 2 mg and placed on sterile Whatman GF/B glass fiber filter disks (6 mm in diameter; Reeve-Angel, Clifton, NJ)

15   to which 30 µl of transduced cells have been added. They are placed upside down on the CAM of 10-day-old chicken embryos through windows made in the egg shells on day 8 of inoculation. The embryos are killed 5 days later by injection of 10% fomalin in PBS. The CAM is excised, fixed in 10% formalin in PBS inverted, and examined under a stereo-microscope. Angiogenesis is assayed by measuring the number and thickness of capillaries

20   beneath the filter. A thick capillary, a middle sized capillary, a small capillary, and 5 minute capillaries are given 3, 2, 1 and 1 points, repectively, and the average number of points is defined as the angiogenic activity. The diameters of tumors on the filters are measured in three dimensions and the tumor size is calculated as $(\pi/6)$ abc mm^3 (a, b, and c: length, width, and height, respectively). Low average number of points and decreased tumor size

25   indicates anti-angiogenic activity.

C.   Determination of Tumor Vascularization

To visualize the vascularization of tumors treated in accordance with methods

30   taught herein, mice are injected with 300 µl of India ink at 4 minutes before sacrifice and the skin is taken and dried. The free blood flow is estimated using 10 µm E-Z TRAC ultraspheres (Interactive Medical Technology, Los Angeles, CA). 5×10^5 of 10 µm microspheres are injected into the left ventricle of a mouse and the tumor tissue harvested after 5 minutes. After dissolving the tumor tissue, the beads present in the tumor are

35   counted and expressed as beads per gram tumor tissue.

D.   Determination of the *Herpes* Simplex Virus Thymidine Kinase Activity

The sensitivity of HSVTK vector transduced cells to gancyclovir may be used to determine the activity of expressed HSVTK expressed in cells treated according to the disclosed methods. Briefly, cells that are tranduced with pTK-3 are seeded into six plates at a density of 2.5 x 10^{6} per plate. In addition, untransduced CT26 and CT26 β-gal (this cell line was transduced with a virus carrying the reporter gene β-galactosidase from *E. coli.*), are also seeded into six plates as controls. Five plates of each cell type are treated twice per day for four consecutive days with medium containing ganciclovir concentrations of 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml, and 6.25 μg/ml. One plate of each cell type is left untreated. Following this treatment, the cells are removed from each dish using trypsin/EDTA, resuspended in DMEM with 10% FBS and counted. Similar protocols can be employed for other pro-drug activating enzymes.

E.   Determination of Folate-Integrin β3 Activity

i.   Cell Surface Folic Acid Binding Assay

MCF-7 stable transfectants are plated and maintained in the same media without G418. Plating densities are adjusted so that cells are 75% confluent for the folic acid binding assays. The form of radiolabeled folic acid used is an iodinated, histamine derivative of folic acid obtained from New England Nuclear. Cells are washed twice with 3 ml ice-cold pH 4.5 saline (10 mM Na-acetate, 150 mM NaCl) to dissociate surface-bound folates from cell surface folate receptor. Cell monolayers are subsequently washed twice with 3 ml ice cold PBS, pH 7.4, to return the pH to neutral. For cell surface-binding of radiolabeled folic acid, cells are incubated in 2 ml of ice-cold DMEM (without FCS), containing iodinated, histamine derivative of folic acid (20,000 cpm total added to 50 nM cold folic acid) and 50 μg/ml BSA for 15 minutes in an ice-H$_2$O bath. To determine specific cell surface binding (cpm/mg protein), parallel experiments are performed in which 1,000-fold excess of cold folic acid is added. The monolayer is washed twice with (with 3 ml each) ice-cold PBS, pH 7.4. Cells are solubilized and supernatant samples are counted in a gamma counter (55B; Beckman Instruments, Inc., Dallas, TX) at ~70% efficiency. Protein concentration in each solubilized sample are determined as described above.

ii.   [^{3}H] Folic Acid Binding Assay

The [^{3}H] folic acid binding assay is performed both by direct binding of [^{3}H] folic acid to membranes and by a solution-phase assay. In the direct binding assay, membrane

5   samples (10-100 μg of protein) are incubated with 3 pmol of [^{3}H] folic acid (40 Ci/mmol) in 10 mM sodium phosphate buffer (pH 7.5)/150 mM NaCl/10 mM EDTA for 30 minutes at 37_C with constant agitation. The membranes are sedimented at 12,000 g for 15 minutes, washed once with the same buffer, dissolved in 10 mM sodium phosphate buffer (pH 7.5)/150 mM NaCl/1% Triton X-100, and subjected liquid scintillation counting.

10   Nonspecific binding of [^{3}H] folic acid is determined for both the assays by performing the assay in each case simultaneously with controls in which the addition of [^{3}H] folic acid is preceded by incubation for 5 minutes at room temperature with 100 pmol of unlabeled folic acid.

15   iii.   Methotrexate Transport Studies

Stable transfectants are plated and cell monolayers are washed twice with 3 ml ice-cold pH 4.5 saline and twice with 3 ml ice-cold PBS, pH 7.4. For internalization of irradiated methotrexate (^{3}H-MTX) cells are incubated in 2 ml of prewarmed (37_C)

20   DMEM (without FCS or other additives), containing 50 μg/ml BSA, and 2 μM [^{3}H] MTX, for 30 minutes at 37_C, 5% CO_2. To determine specific MTX internalization (pmol/mg protein), parallel experiments are performed in the presence of 500-fold excess cold MTX. To differentiate transport mediated by human folate receptor (hFR) from the reduced folate transporter, parallel experiments are run in the presence of molar (100-fold) excess of cold

25   folic acid which inhibits transport via hFR. The monolayer is washed once with 2 ml ice-cold PBS (pH 7.5), once with 2 ml pH 4.5 saline to remove surface-bound ^{3}H-MTX, and finally with 2 ml of ice-cold PBS (pH 7.5). Cells are solubilized and samples (500 μl) are added to 10 ml of liquid scintillation cocktail and counted on a liquid scintillation counter (Tri-carb; Packard Instrument Co., Inc., Downers Grove, IL) at ~50% efficiency. Protein

30   concentration in each solubilized sample is determined as described above

F.   Determination of Integrin B3 Activity

i.   Peptides

35

A 16-amino acid fibrinogen γ chain peptide (K16), having the sequence KYGGHHLGGAKQAGDV, is prepared by solid phase synthesis on an Applied

Biosystems model 430 peptide synthesizer (Foster City, CA) using phenylacetamidomethyl resins and t-butoxycarbonyl amino acids purchased from Applied Biosystems. The peptide is analyzed for homogeneity by high performance liquid chromatography using a C18 μBondapak column with a linear gradient of 0-60% acetonitrile in 0.1% trifluoroacetic acid

5   and is found to be >85% homogeneous. The peptide is dissolved in PBS (0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.3) and radioiodinated by a modified lactoperoxidase-glucose oxidase method (D'Souza, et al., J. Biol. Chem. 263:3943, 1988 and Lam, et al., J. Biol. Chem. 262:947, 1987). Glucose (40 μg in 80 μl of 0.2 M sodium phosphate, pH 7.4), carrier-free Na^{125} I (15 m Ci), and Enzymobead reagent (Bio-Rad,

10   Hercules, CA) are added to 10-12 mg of the peptides. The iodinated peptide is separated from free Na^{125} I by gel filtration on a Bio-Gel P-2 column (Bio-Rad, Hercules, CA). The concentration of the labeled peptide is determined by absorbance at 280 nm using in extinction coefficient derived from its amino acid composition. ·

15   ii.   Platelet Binding and Cross-Linking

          Platelets are isolated from fresh human blood by differential centrifugation followed by gel filtration on Sepharose 2B in divalent ion-free Tyrode's buffer, pH 7.3, containing 0.1% bovine serum albumin. Briefly, platelets are suspended at 4 x 10^8/ml in divalent ion-

20   gree Tyrode's-albumin buffer. Ca^{2+} is added to a final concentration of 1 mM. The platelet stimuli used are 10 μM ADP, 0.5 unit/ml α-thrombin, or 100 mM PMA. The radiolabeled K16 peptide is added at a concentration of 30 μM, and binding proceeded for 45 minutes at 22_C. The primary cross-linking agent is bis(sulfosuccinimidyl)suberate (BS^3) (Pierce Chemical Co., Rockford, IL). The cross-linking reactions are terminated after 10 minutes

25   at 22_C by addition of 10 mM Tris, pH 7.0. The cell-bound ligand is recovered by centrifugation through 20% sucrose, and the cells are extracted in PBS containing 1% Nonidet P-40 and 10 mM N-ethylmaleimide (Sigma, St. Louis, MO). Extracted proteins are precipitated with 10% trichloroacetic acid, and the pellet obtained after centrifugation is washed three times with cold 85% ethanol.

30   Polyacrylamide gel electrophoresis is performed in the presence of sodium dodecyl sulfate in vertical slab gels in the buffer system of Laemmli (Nature 227:680, 1970). Gels of varying percentages are used. For initial analysis of cross-linked samples 7.5% gels are run under nonreducing conditions. Gels utilized for amino acid sequencing are 10-20% gradient gels. Samples in Laemmli sample buffer are treated with 5% 2-mercaptoethanol

35   for disulfide bond reduction. Analytical gels are dried, and autoradiograms are developed with Kodak X-Omat AR films. Molecular weight is estimated on the basis of electrophoretic mobility relative to prestained standards obtained from Diversified Biotech.

The relative mobility (R_F) is determined by measuring the migration of the ^{125}I-K16-GPIIb band relative to the migration of the ovalbumin marker protein in the same gel.

Example 6

Formulation of the Retroviral Vector

A.   Lactose Formulation

Crude recombinant retrovirus is obtained from a Celligan bioreactor (New Brunswick, New Brunswick, NJ) containing DA cells transformed with the recombinant retrovirus (U.S.S.N. 07/395,932) bound to the beads of the bioreactor matrix. The cells release the recombinant retrovirus into the growth media that is passed over the cells in a continuous flow process. The media exiting the bioreactor is collected and passed initially through a 0.8 micron filter then through a 0.65 micron filter to clarify the crude recombinant retrovirus. The filtrate is concentrated utilizing a cross flow concentrating system (Filtron, Boston, MA). Approximately 50 units of DNase (Intergen, New York, NY) per ml of concentrate is added to digest exogenous DNA. The digest is diafiltrated using the same cross flow system to 150 mM NaCl, 25 mM tromethamine, pH 7.2. The diafiltrate is loaded onto a Sephadex S-500 gel column (Pharmacia, Piscataway, NJ), equilibrated in 50 mM NaCl, 25 mM tromethamine, pH 7.4. The purified recombinant retrovirus is eluted from the Sephadex S-500 gel column in 50 mM NaCl, 25 mM tromethamine, pH 7.4.

The formulation buffer containing lactose was prepared at a 2x concentrated stock solution. The formulation buffer contains 25 mM tromethamine, 70 mM NaCl, 2 mg/ml arginine, 10 mg/ml human serum albumin (HSA), and 100 mg/ml lactose in a final volume of 100 mls at a pH 7.4.

The purified recombinant retrovirus is formulated by adding one part 2x lactose formulation buffer to one part S-500 purified recombinant retrovirus. The formulated recombinant retrovirus can be stored at -70°C to -80°C or dried.

The formulated retrovirus is lyophilized in an Edwards Refrigerated Chamber (3 Shelf RC3S unit) attached to a Supermodulyo 12K freeze dryer (Edwards High Vacuum, Tonawanda, NY). When the freeze drying cycle is completed, the vials are stoppered under a vacuum following a slight nitrogen gas bleeding. Upon removal, vials are crimped with aluminum seals.

In the given lactose study, formulated liquid product was stored at both -80°C and at -20°C cycling freezer. In Figure 20, viral infectivity of these samples were compared to the viral infectivity of lyophilized samples. The lyophilized samples were stored at -20°C, refrigerator temperature and room temperature. Activity of the samples upon reconstitution are determined by titer assay.

The lyophilized recombinant retrovirus is reconstituted with 1.0 ml water. The infectivity of the reconstituted recombinant retrovirus is determined by a titer activity assay. The assay is conducted on HT 1080 fibroblasts or 3T3 mouse fibroblast cell line (ATCC CCL 163). Specifically, 1.0×10^5 cells are plated onto 6 cm plates and incubated overnight at 37°C, 10% CO_2. Ten microliters of a dilution series of reconstituted recombinant retroviruses are added to the cells in the presence of 4 µg/mL polybrene (Sigma, St. Louis, MO) and incubated overnight at 37°C, 10% CO_2. Following incubation, cells that have been transduced with a recombinant vector which encodes the *neo* resistance gene are selected for neomycin resistance in G418 containing media and incubated for 5 days at 37°C, 10% CO_2. Following initial selection, the cells are re-fed with fresh media containing G418 and incubated for 5 to 6 days. After final selection, the cells are stained with Commassie blue for colony detection. The titer of the sample is determined from the number of colonies, the dilution and the volume used.

Figure 20 demonstrates that storage in lyophilized form at -20°C to refrigerator temperatures retains similar viral activity as a recombinant retrovirus stored in liquid at -80° to -20°C permitting less stringent temperature control during storage.

B.   Mannitol Formulation

The recombinant retrovirus utilized in this example was purified as described in A above.

The formulation buffer containing mannitol was prepared as a 2x concentrated stock solution. The formulation buffer contains 25 mM tromethamine, 35 mM NaCl, 2 mg/ml arginine, 10 mg/ml HSA and 80 mg/ml mannitol at a final volume of 100 mls at a pH 7.4.

The purified recombinant retrovirus is formulated by adding one part mannitol formulation buffer to one part S-500 purified recombinant retrovirus. The formulated recombinant retrovirus can be stored at this stage at -70°C to -80°C or dried.

The formulated retrovirus is dried in an Edwards Refrigerated Chamber (3 Shelf RC3S unit) attached to a Supermodulyo 12K freeze dryer. When the freeze drying cycle is completed, the vials are stoppered under a vacuum following nitrogen gas bleeding to 700 mbar. Upon removal, vials are crimped with aluminum seals.

In the given mannitol study, formulated liquid product was stored at both -80°C and at -20°C in cycling freezers. The viral infectivity of these samples were compared to the viral infectivity of lyophilized samples, Figure 21. The lyophilized samples were stored at -20°C, refrigerator temperature and room temperature. Activity of the samples upon reconstitution are determined using the titer assay described in A above.

Figure 21 demonstrates that storage in lyophilized form at -20°C to refrigerator temperature retains significant viral activity as compared to recombinant retrovirus stored in liquid at -80°C or -20°C, permitting less stringent temperature control during storage.

C.   Trehalose Formulation

The recombinant retrovirus utilized in this example was purified as described in A above.

The formulation buffer containing trehalose was prepared as a 2x concentrated stock solution. The formulation buffer contains 25 mM tromethamine, 70 mM NaCl, 2.0 mg/ml arginine, 10.0 mg/ml HSA and 100 mg/ml trehalose at a final volume of 100 mls at a pH 7.2.

The purified recombinant retrovirus is formulated by adding one part trehalose formulation buffer to one part S-500 purified recombinant retrovirus. The formulated recombinant retrovirus can be stored at this stage at -70°C to -80°C or dried.

The formulated retrovirus is dried in an Edwards Refrigerated Chamber (3 Shelf RC3S unit) attached to a Supermodulyo 12K freeze dryer. When the freeze drying cycle is completed, the vials are stoppered under a vacuum following nitrogen gas bleeding to 700 mbar. Upon removal, vials are crimped with aluminum seals.

In the given trehalose study, formulated liquid product was stored at both -80°C and at -20°C in cycling freezers. The viral infectivity of these samples was compared to the viral infectivity of lyophilized samples, Figure 22. The lyophilized samples were stored at -20°C, refrigerator temperature and room temperature. Activity of the samples upon reconstitution are determined using the titer assay as described in A above.

Figure 22 demonstrates that storage in lyophilized form at -20°C to refrigerator temperature retains similar viral activity as compared to recombinant retrovirus stored in liquid at -80°C to -20°C permitting less stringent temperature control during storage.

Viral infectivity of liquid formulated recombinant retrovirus samples stored at -80°C was compared to viral infectivity of lyophilized formulated recombinant retrovirus stored at -20°C. Initially, a bulk of recombinant retrovirus was received and formulated in four different ways as shown below. The formulated recombinant retrovirus was then frozen in bulk for 1.5 months subsequent to being quick thawed and freeze dried. Positive controls

were stored at -80°C for comparison with lyophilized samples which were stored at -20°C after freeze-drying. The formulations are listed below:

5

Formulation	Sugar Concentration (mg/ml)	Buffer Concentration (mM tromethamine)	Salt Concentration (mM NaCl)	Arginine Concentration (mg/ml)	Human Serum Albumin Concentration (mg/ml)
Mannitol	40	25	25	1	5
Lactose	40	25	75	1	5
Sucrose	50	25	60	1	5
Trehalose	50	25	60	1	5

In the graphs of Figure 23, the y-axis on each of the 4 graphs (A, B, C, D) represent the normalized titer. At an initial time point after lyophilization, t = 0, a titer value was established for both the -80°C liquid sample and the -20°C lyophilized sample. At each

10 time point of the stability study, the titer obtained was divided by the zero time point titer value and the percentage of original entered onto the graph.

The data demonstrates that post-lyphilization activity is maintained in the lyophilized sample (stored at -20°C) relative to the liquid sample (stored at -80°C). The formulated lyophilized recombinant retrovirus was stored in a -20°C freezer (a frost-free

15 cycling freezer). Comparison to the formulated liquid recombinant retrovirus stored at -80°C indicates the lyophilized form permits less stringent control of storage conditions.

D.   Sindbis Formulation

20   Crude recombinant alphavirus vector is obtained from a Celligan bioreactor containing packaging cells transfected or transduced with the recombinant alphavirus vector, and bound to the beads of the bioreactor matrix. The cells release the recombinant alphavirus vector into the growth media that is passed over the cells in a continuous flow process. The media exiting the bioreactor is collected and passed initially through a 0.8

25 micron filter then through a 0.65 micron filter to clarify the crude recombinant alphavirus vector. The filtrate is concentrated utilizing a cross flow concentrating system. Approximately 50 units of DNase per ml of concentrate is added to digest exogenous DNA. The digest is diafiltrated using the same cross flow system to 150 mM NaCl, 25 mM tromethamine, pH 7.2. The diafiltrate is loaded onto a Sephadex S-500 gel column,

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equilibrated in 50 mM NaCl, 25 mM tromethamine, pH 7.4. The purified recombinant alphavirus vector is eluted from the Sephadex S-500 gel column in 50 mM NaCl, 25 mM tromethamine, pH 7.4.

The formulation buffer containing lactose is prepared as a 2X concentrated stock
5   solution. The formulation buffer contains 25 mM tromethamine, 70 mM NaCl, 2 mg/ml arginine, 10 mg/ml HSA, and 100 mg/ml lactose in a final volume of 100 mls at a pH 7.4.

The purified recombinant alphavirus vector is formulated by adding one part 2X lactose formulation buffer to one part S-500 purified recombinant alphavirus vector. The formulated recombinant alphavirus vector can be stored at -70°C to -80°C or dried.

10   The formulated alphavirus vector is lyophilized in an Edwards Refrigerated Chamber (3 Shelf RC3S unit) attached to a Supermodulyo 12K freeze dryer. When the freeze drying cycle is completed, the vials are stoppered under a vacuum following a slight nitrogen gas bleeding. Upon removal, vials are crimped with aluminum seals. The lyophilized recombinant retrovirus is reconstituted with 1.0 ml water or other
15   physiologically acceptable diluent.

Example 7

20

Administration Protocols

A.   Mice

25   i.   Administration of Recombinant Retroviral Vector Transduced Cells

              a.   Determination of the Effect of Ganciclovir on CT26 with or without TK-3

Colon tumor cells (CT 26) (Brattain, Baylor College of Mediine, Houston, TX) are
30   transduced with DA/TK-3, a G-pseudotyped TK-3 vector. Twenty-four hours after adding the viral supernatant, the CT26 cells are placed under G-418 selection (450 µg/ml). After 10 days incubation, a G-418 selected pool is obtained and designated CT26 TKneo. (CT26 TKneo), CT26 TKneo cells were seeded into six 10 cm² plates at a density of 2.5 X 10^6 per plate. As contrasts, each of two other cell types, CT26 and CT26 β-galactosidase,
35   (β-gal), (this cell line was transduced with a virus carrying the reporter gene β-gal from E. coli.), were also seeded into six 10 cm² plates as controls. Five plates of each cell type were treated twice per day for four consecutive days with medium containing ganciclovir

concentrations of 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml and 6.25 µg/ml. One plate of each cell type was left untreated. Afterwards, the cells were removed from each dish using trypsinedta, resuspended in DMEM with 10% FBS and counted. The data in Figure 24 shows that even the lowest dose of ganciclovir had a dramatic cytotoxic effect on the CT26 TK*neo* cells. This dose of ganciclovir (6.25 µg/ml) or even the next higher dose (12.5 µg/ml) did not have an effect on either the CT26 or CT26 β–gal cells. However, beginning at a ganciclovir dose of 25 µg/ml, a dose-dependent decrease in cell growth could be seen, although CT26 TK *neo* cells were always more sensitive to the drug.

b.   Determination of a Ganciclovir Dose for the Treatment of Mice
         Injected with CT26 TK*neo* Cells

In order to test whether *in vivo* transduction of a murine tumor could be used to treat the disease, an experiment was performed to determine the optimal concentration of ganciclovir necessary to eliminate a tumor that was transduced and selected *in vitro* to assure 100% transduction. Twelve groups of 3 Balb/c (Harlan Spague Dawley, Indianapolis, IN) mice each are injected with 2.0 X 10^5 CT26 TK*neo* cells. Six groups of mice are injected with these cells intraperitoneally (I.P.) and six groups of mice are injected subcutaneously (S.C.). Two other groups of 3 mice each are injected with 2.0 X 10^5 unmodified CT26 cells (as a control) either I.P. or S.C..

Ten days after the injection of the CT26 or CT26 TK*neo* cells into these groups of mice, several concentrations of ganciclovir treatment are initiated. Each dose regimen consists of 2 daily AM and PM I.P. injections of ganciclovir. The experiment is summarized in Table A below.

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TABLE A

Group	Innoculum	Injection Route	Concentration of Ganciclovir (mg/Kg)
1	CT26	I.P.	0
2	CT26 TK*neo*	I.P.	0
3	CT26 TK*neo*	I.P.	15.63
4	CT26 TK*neo*	I.P.	31.25
5	CT26 TK*neo*	I.P.	32.5
6	CT26 TK*neo*	I.P.	125.0
7	CT26 TK*neo*	I.P.	250.0
8	CT26 TK*neo*	I.P.	500.0
9	CT26	S.C.	0
10	CT26 TK*neo*	S.C.	0
11	CT26 TK*neo*	S.C.	15.63
12	CT26 TK*neo*	S.C.	31.25
13	CT26 TK*neo*	S.C.	62.5
14	CT26 TK*neo*	S.C.	125.0
15	CT26 TK*neo*	S.C.	250.0
16	CT26 TK*neo*	S.C.	500.0

After 5 days, all of the mice in the 125 mg/Kg, 250 mg/Kg and 500 mg/Kg treated groups were dead due to the toxic effects of ganciclovir. Mice in the 15.63 mg/Kg, 31.25 mg/Kg and 62.5 mg/Kg treated groups were treated for an additional 7 days and were able

5   to tolerate the treatment. Tumor measurements were made for 23 days (Figure 25). CT26TK *neo* grew only slightly slower than unmodified CT26. Complete tumor regression was seen in the groups of mice treated with the 62.5 mg/Kg regimen. Partial tumor regression was seen in the 31.25 mg/Kg treated groups. Little or no effect was seen in the 15.63 mg/Kg treated groups as compared to the 2 untreated control groups. Even though

10 there was some toxicity observed in the 62.5 mg/Kg groups, it was not life threatening and reversible upon the discontinuation of the treatments so this concentration was used for future studies (Figure 25). After 24 days, the I.P. injected animals were sacrificed and evaluated. As seen in Figures 26 and 27 the optimal concentration for anti-tumor effect was similar whether the tumor was grown I.P. or S.C..

15

ii.   Administration of Gene Delivery Vehicles

      a.   Transmission and Expression of Vector Encoded Genes in Tissue
            Culture

5

The transmission and expression of the specific vector encoded genes is tested by transduction of tissue culture cells, followed by testing of expression in these cells. 10^6 HT1080 cells (a human fibrosarcoma line) or other appropriate cells (*e.g.* other human tumor derived lines such as MCF-7, a breast tumor derived cell line; primary human

10   endothelial cells, HUVEC; mouse tumor cell lines such as the colon tumor line CT26, the fibrosarcoma line L33, the melanoma cell line B16, the breast tumor cell line Tg-6-2, the neuroblastoma cell line C1300; mouse endothelial cells or cell lines such as the SVEC cell line in a 10 cm dish are treated with the gene delivery vector at a multiplicity of cell transduction units (*e.g.* colony forming units) of 1-10. After 2-6 days the cells are harvested

15   and the appropriate assays used to test for expression of the transferred gene. If appropriate antibodies are available, assays described in Examples 4A and 4B can be used. Examples of specific activity assays are given for Tissue Factor (Example 5A), general antiangiogenic factors (Example 5B), Herpes thymidine kinase (Example 5D), folate-integrin B3 (Example 5E).

20

      b.   Administration to Animals and Detection of Activity and Biological
            Effects

The major issues in the use of the invention is to achieve sufficient expression of the

25   novel effector protein such as the Russel viper venom protein, tissue factor, prodrug activating enzyme, or metabolism altering protein such as uricase, in the tumor or the peritumoral vascular system, without having unacceptable toxic effects due to significant expression elsewhere.

The following mouse models are used to assess this: BalB/c mice plus the L33 or

30   CT26 cell lines; C57B/6 plus B16 cells;FVB/N plus TG-6-2 cells; C3H mice plus C1300 or SVEC cells;   BalB/c nu/nu mice plus any of these, or plus human tumors such as the melanoma lines DM252, DM 6, DM92 (*see* U.S.S.N. 08/032846) or MCF-7 cells.

Tumor cells are injected at an appropriate dose (typically 2×10^5 cells) either subcutaneously, I.V. for the B16 lung metastases model, or intra-splenically for the CT26

35   liver metastases model. Groups (1-10 mice) of the injected mice are maintained until palpable tumors (1-4 mm in diameter) are apparent for subcutaneous tumors, or for the length of time that leads to easily detectable metastases, upon necropsy, in the metastases

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models. This is typically 7 to 14 days. At this time the tumor vascularization can be visualized using the techniques in Example 5C and various routes of administration examined to determine the one that gives best flow into the tumors.

The vector preparations are injected at the previously determined I.V. or peritumoral site at doses of 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, or 10^{11} vector units (e.g. colony forming units, cfu) with or without transduction enhancers such as polybrene (1-8 µg/ml) or DEAE dextran (2 - 30 µg/ml). Injections are given daily for 1, 2, 3, 4, 5, 6, or 7 days, and 2 to 7 days after the last injection, the tumors are excised and assayed for acquisition of the activity of the delivered gene (see, for example, Example 4C, 4D, and Example 5). Controls are mice injected with an irrelevant, inert vector (e.g. encoding human gamma interferon which is inactive in mice). The vector encodes genes of the types described in the detailed description and in the case of a retroviral vector carries a non-specific envelope such as the amphotropic or xenotropic envelopes, and relies on the paucity of replicating cells in the general vascular endothelium, and the over-representation of such replicating cells in sites of neovascularization such as tumors. Alternatively the vector carries an appropriate targeting ligand that avoids non-specific adsorption, and allows binding and entry into the target cell type. The specificity of the system may also be controlled by the inclusion of appropriate expression control elements

The same administration protocols or a smaller number chosen on the bases of the above experiments or other criteria, are used to measure the antitumor effect in these mouse models. The endpoints are tumor size and growth/regression over time, time to death, survival, or other appropriate marker (e.g. number of metastases in those models).

c.　　Administration to Humans

Patients are treated with the same vectors as the mouse models or the same nucleic acid backbone carried in a vector that is resistant to human complement (see, for example, the companion application "Production and Administration of High Titer Recombinant Retroviruses") Patients with tumor masses that are not removable surgically (e.g. brain, prostate, cervical, ovarian, bladder), for which removal requires simultaneous removal of large amounts of irreplaceable healthy tissue (e.g. amputation of limbs or removal of whole organs), or where metastasis may have taken place (e.g. colon metastasis to the liver, melanoma metastasis to the brain, other subcutaneous sites and the lung), can be treated.

Patients receive doses of 10^6, 10^7, 10^8, 10^9, 10^{10}, or 10^{11} vector units I.V., intra-arterially, in the local vasculature or peritumorally in a volume of 0.1 to 3 ml preferably. The vector carries a gene that encodes a clotting enhancer or other gene chosen from the

types described above. If the gene is one that converts a non-toxic precursor (prodrug) into a toxic product , the prodrug is administered at doses defined in the Physicians Desk Reference or those predicted from animal experiments at times of between 1 to 30 days after the last administration of vector.

5   The vectors are administered from 1 to 20 times at intervals of 1 to 15 days and the patient status is monitored by following normal clinical parameters and monitoring tumor sizes by radiography, mri scans, pet scans or other conventional means

10   Example 8

Targeting Recombinant Retroviral Vector to Melanoma Cells

The following example describes the use of the coupled targeting element Ab-biotin

15   to target the coupled retroviral vector particle-avidin to a specific cell type.   Generally, biotinylated melanocyte stimulating hormone (MSH) is first injected into the patient. After a period of time (up to 3 days) after which non-specific binding has decayed and only specific ligand complexes remain, a vector expressing avidin on its surface is injected. The high affinity of avidin for biotin focuses the vector to the target tissue.

20   Briefly, melanocyte-stimulating hormone (MSH) is a 13 amino acid peptide that is specifically recognized by a receptor on melanocytes. MSH has a receptor affinity (K_D) in the range of 10^{-8}M.

A.   Construction of an Expression Cassette Backbone, pH CMV-PA

25

A vector is first created in order to form the backbone for both the *gag/pol* and *env* expression cassettes. Briefly, pBluescript SK⁻ phagemid (Stratagene, San Diego, CA, GenBank accession number 52324, referred to as "SK⁻") is digested with Spe I and blunt ended with Klenow. A blunt end Dra I fragment of SV40 (Fiers, *et al., Nature 273*:113-

30   120, 1978) from Dra I (bp 2,366) to Dra I (bp 2,729) is then inserted into SK⁻, and a construct isolated in which the SV40 late polyadenylation signal is oriented opposite to the LacZ gene of SK⁻. This construct is designated SK-SV40A.

A human cytomegalovirus major immediate early promoter (HCMV-MIE; Boshart, *et al., Cell 41*:521-530, 1985) (Hinc II, bp 140 to EagI, bp 814) is isolated after digestion

35   with Hinc II and Eag I, and the Eag I site blunt ended. The 674 blunt ended fragment is ligated into SK-SV40A. The final construct, designated pHCMV-PA is then isolated (*see*

Figure 28). This construct contains the HCMV promoter oriented in opposite orientation to the LacZ gene, and upstream from the late polyadenylation signal of SV40.

B.   Construction of pCMV-env^{eco}

pCMV-env^{eco} is created by inserting the Xba I-Nhe I fragment of MoMLV (bp 5,766 through bp 7,845 of MoMLV) into pCMV-PA (Example 2A) expression vector. Briefly the Xba I-Nhe I envelope fragment is isolated from pMLV-K (Miller *et al., J. Vir.* 49:214, 1988) on an agarose gel. The fragment is then blunt-ended with T4 polymerase using standard methods, ligated into pCMV-PA (Example 2), and digested at the Eco RV and Sma I sites. The product in the correct orientation has a CMV MIE promoter followed by the complete ecotropic envelope coding sequence and an SV40 polyadenylation signal.

C.   Creation of Avidin-Envelope Chimera

A portion of avidin DNA (sequence #27, GenBank # CHKAVIR) from bp 116 through bp 499 is incorporated into the MoMLV ecotropic envelope construct pCMV-env^{eco}. Briefly, the following oligonucleotide is generated as follows:

(Sequence ID No.: 51)

5'-GCT AGA ATA TCA AGC CAG AAA GTG CTC GCT GAC TGG GAA ATG
GAC CAA CGA TCT GGG CTC CAA CAT GAC CAT CGG GGC TGT GAA
CAG CAG AGG TGA ATT CAC CCT GCG CAC ACA GAA GGA GCG GTG
CAA CAC-3'

The oligonucleotide is used to modify single stranded pCMV-env^{eco} by the method of Kunkle (*PNAS 82*:488, 1985). This modification replaces a portion of the variable A region of envelope (Battini, *et al., J. Virol. 66*:1468-1475, 1992) with the sequence of the oligonucleotide. The product is then digested with EcoR1 and partially digested with Fsp I. The Eco RI-Fsp I fragment of avidin (bp 198 through bp 485) is ligated into the vector. The final product is a plasmid containing CMV promoter, hybrid eco-avidin envelope and SV40 polyadenylation signal, called pCMV-env^{eco-avidin}.

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D.   Biotinylated MSH

The MSH peptide S-Y-S-M-E-H-F-R-W-G-L-P-V-NH$_2$ is synthesized (Chiron Corp., Emeryville, CA), and biotinylated with NHS-Biotin (Pierce, Rockford, IL) according to the manufacturer's instructions.

E.   Generation of Marker Recombinant Retroviral Vector Displaying Avidin

The β-galactosidase encoding marker recombinant retroviral vector, CBß-gal is cotransfected into cell line 293 2-3 (see U.S.S.N. 07/800,921) along with pCMV-env^{eco}-avidin. Alternatively, equivalent vectors encoding luciferase, green fluorescent protein (GFP) or other markers can be used. Clones are selected (with G418) and screened for high production of RNA containing particles and screened for surface expression of avidin using ^{3}H-biotin binding. Vector particles containing avidin are tested utilizing ^{14}C-biotin (Amersham, Chicago, IL) and a sucrose gradient.

F.   In vitro Targeting

Human melanoma cells, DM252, DM6, DM92 are grown in appropriate medium. The specificity of biotinylated MSH binding to target cells is tested by addition of avidin-fluorescein and fluorescence microscopy. Transduction of eco-avidin CBß-gal is tested either by staining or by G418 selection (see U.S.S.N. 08/032,846), and the efficiency of tranduction compared to non-melanoma cells such as HT1080 human fibrosarcoma cells.

G.   In vivo Targeting

Nude mice are implanted with one or more of the following human melanoma cell lines: DM252, DM6, DM92 (see U.S.S.N. 08/032,846) in the peritoneal cavity. Targeting is determined by first injecting biotin-MSH into the mouse, followed by injection of 1 x 10^5 to 1 x 10^8 cfu eco-avidin CB ß-gal recombinant retroviral vectors. Targeting is assessed by subsequently dissecting the melanoma tissue, and staining for ß-gal, or assaying for luciferase activity in the melanoma and mouse tissue. As a control, the same vectors encapsidated in the pCMV-env^{eco} transfection of 293-2-3 cells, and with no added envelope plasmids, are injected into mice in parallel, and the tissues of these mice are assayed.

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H.   *In vivo* Targeting - Humans

The data generated in the mouse system is used to determine the protocol for administration in patients. The patients receive three to twenty doses of vector encoding the desired gene I.P., I.V., inter-arterial (I.A.), or into the lymphatic system. The dosage will range from about 1.0×10^6 to about 1.0×10^{10} cfu of the gene delivery vehicle given I.V., I.A., I.P., or into the lymphatics.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (i) APPLICANT: CHIRON VIAGENE INC.

   (ii) TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR TREATMENT OF
                                SOLID TUMORS IN VIVO

   (iii) NUMBER OF SEQUENCES: 42

   (iv) CORRESPONDENCE ADDRESS:
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      (C) CITY: Emeryville
      (D) STATE: California
      (E) COUNTRY: U.S.A.
      (F) ZIP: 94662-8097

   (v) COMPUTER READABLE FORM:
      (A) MEDIUM TYPE: Floppy disk
      (B) COMPUTER: IBM PC compatible
      (C) OPERATING SYSTEM: PC-DOS/MS-DOS
      (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

   (vi) CURRENT APPLICATION DATA:
      (A) APPLICATION NUMBER: PCT - Unassigend
      (B) FILING DATE: Even Date Herewith
      (C) CLASSIFICATION:

   (viii) ATTORNEY/AGENT INFORMATION:
      (A) NAME: Kruse, Norman J.
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      (C) REFERENCE/DOCKET NUMBER: 1154.100

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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 42 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TCGAGCGCTA TGCATGTTTA AACGCGTGCG GCCGCACGTG AT   42

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 41 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGATCACGTG CGGCCGCACG CGTTTAAACA TGCATAGCG C   41

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAGAGATGGG GGAGGCTAAC TGAG   24

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATCCTCAGT TAGCCTCCCC CATCTCTC   28

(2) INFORMATION FOR SEQ ID NO:5:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 42 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TATATTCTAG ATTTTTTTTT TTTTTTTTTT TTTTTTGAAA TG   42

(2) INFORMATION FOR SEQ ID NO:6:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 48 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TATATGGGCC CGATTTAGGT GACACTATAG ATTGACGGCG TAGTACAC   48

(2) INFORMATION FOR SEQ ID NO:7:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 23 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTGGCAACCG GTAAGTACGA TAC   23

(2) INFORMATION FOR SEQ ID NO:8:

   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 21 base pairs

    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: cDNA

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATACTAGCCA CGGCCGGTAT C   21

(2) INFORMATION FOR SEQ ID NO:9:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 21 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: cDNA

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TCCTCTTTCG ACGTGTCGAG C   21

(2) INFORMATION FOR SEQ ID NO:10:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 21 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: cDNA

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACCTTGGAGC GCAATGTCCT G   21

(2) INFORMATION FOR SEQ ID NO:11:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 21 base pairs
    (B) TYPE: nucleic acid.
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: cDNA

(2) INFORMATION FOR SEQ ID NO:11:

CCTTTTCAGG GGATCCGCCA C   21

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTGGCGGATC CCCTGAAAAG G   21

    (2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGGGCCGTGT GGTCGTCATG   20

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGGGTCTTCA ACTCACCGGA C   21

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 22 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CAATTCGACG TACGCCTCAC TC   22

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 22 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GAGTGAGGCG TACGTCGAAT TG   22

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 33 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TATATAGATC TAATGAAAGA CCCCACCTGT AGG   33

(2) INFORMATION FOR SEQ ID NO:18:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 40 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCAATCCCCG AGTGAGGGGT TGTGGGCTCT TTTATTGAGC   40

(2) INFORMATION FOR SEQ ID NO:19:

    (i) SEQUENCE CHARACTERISTICS:

- 115 -

            (A) LENGTH: 35 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CCACAACCCC TCACTCGGGG ATTGACGGCG TAGTAC   35

(2) INFORMATION FOR SEQ ID NO:20:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 23 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTGGCAACCG GTAAGTACGA TAC   23

(2) INFORMATION FOR SEQ ID NO:21:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 33 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TATATAGATC TAATGAAAGA CCCCACCTGT AGG   33

(2) INFORMATION FOR SEQ ID NO:22:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 22 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GGTAACAAGA TCTCGTGCCG TG   22

(2) INFORMATION FOR SEQ ID NO:23:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 47 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TATATGCGGC CGCTTTCTTT TATTAATCAA CAAAATTTTG TTTTTAA   47

(2) INFORMATION FOR SEQ ID NO:24:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 48 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TATATGAGCT CTTTTTTTTT TTTTTTTTTT TTTTTTGAAA TGTTAAAA   48

(2) INFORMATION FOR SEQ ID NO:25:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 34 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TATATCTCGA GGGTGGTGTT GTAGTATTAG TCAG   34

(2) INFORMATION FOR SEQ ID NO:26:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 41 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TATATGCGGC CGCTCTAGAT TACAATTTGG ACTTTCCGCC C   41

(2) INFORMATION FOR SEQ ID NO:27:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 32 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCGAGCACGT GTTTAAACGC GTGATCAGGC CT   32

(2) INFORMATION FOR SEQ ID NO:28:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 32 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CTAGAGGCCT GATCACGCGT TTAAACACGT GC   32

(2) INFORMATION FOR SEQ ID NO:29:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CGGCGCGCCG AGCT   14

(2) INFORMATION FOR SEQ ID NO:30:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 44 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CGCGCGGGCC CTGTGACATT GAATAGAGTG AGGGTCCTGT TGGG   44

(2) INFORMATION FOR SEQ ID NO:31:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 45 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AAAGGTTTCA CATTTGTAGC TTGCTGTGTC ATTGCGATCT CTACG   45

(2) INFORMATION FOR SEQ ID NO:32:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 45 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GTGGTCCTAA ATAGTTCACT CTATTCAATG TCACACTCGA GCCGG   45

(2) INFORMATION FOR SEQ ID NO:33:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 38 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TATATGTTTA AACCCGCCAA CTGGTAGACA TGGAGACC   38

(2) INFORMATION FOR SEQ ID NO:34:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 38 base pairs
      (B) TYPE: nucleic acid

    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ATATAGTTTA AACTCCAACA GTGCTTCCTT TATGAAAC   38

(2) INFORMATION FOR SEQ ID NO:35:

   (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 38 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TATATGTTTA AACCACCAGA GAACCCACCA TGGCCCCC   38

(2) INFORMATION FOR SEQ ID NO:36:

   (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 38 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

ATATAGTTTA AACCCACTCC GGGCAGGATT CAGGCTAT   38

(2) INFORMATION FOR SEQ ID NO:37:

   (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 40 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GGTGGAGGTG GAAGCGCTCA GCGGATGACA ACACAGCTGC   40

(2) INFORMATION FOR SEQ ID NO:38:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 38 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

ATATAGTTTA AACAGGTAAA AGGAGGTCAG CTGAGCAG   38

(2) INFORMATION FOR SEQ ID NO:39:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 45 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

TATATGTTTA AACCACCATG GTCAGTCCCC AGAGGATTGC ACTCC   45

(2) INFORMATION FOR SEQ ID NO:40:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 45 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

TGAGCGCTTC CACCTCCACC GAGCTTTCGC ATCTGGGTGG CCTGC   45

(2) INFORMATION FOR SEQ ID NO:41:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 38 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TATATGTTTA AACAGACATG GCTCAGCGGA TGACAACA   38

(2) INFORMATION FOR SEQ ID NO:42:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 46 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TGCAATCCTC TGGGGACTGA CCCTCGCCAC CTCCTCATTG GGGTTG   46

(2) INFORMATION FOR SEQ ID NO:43:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 25 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GTCAGTCCCC AGAGGATTGC ACTCC   25

(2) INFORMATION FOR SEQ ID NO:44:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 131 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

ATATAGTTTA AACTCAGCTG AGCAGCCACA GCAGCATTAG   40
GGCCAGGCTA AGCAGGAAAG GCCAGGCTGC CCAGGGCCCA   80
GCCCCACTCA TGGCTGCAGC ATAGAAGAGC TTTCGCATCT   120
GGGTGGCCAG C   131

(2) INFORMATION FOR SEQ ID NO:45:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

TATATACGCG TCCCCCCCCC CCCCAACG   28

(2) INFORMATION FOR SEQ ID NO:46:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 34 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TATATACGCG TCTTACAATC GTGGTTTTCA AAGG   34

(2) INFORMATION FOR SEQ ID NO:47:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TATATGCGGC CGCATGCAGA TGTCTCCAGC   30

(2) INFORMATION FOR SEQ ID NO:48:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 31 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single

- 124 -

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

TATATGCGGC CGCCCCAAGG TAGTGAACCG G   31

(2) INFORMATION FOR SEQ ID NO:49:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 47 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

TATATGCGGC CGCACCACCA CCATGAATAG AGGATTCTTT AACATGC   47

(2) INFORMATION FOR SEQ ID NO: 50

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 34base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

TATATGCGGC CGCTCATCTT CGTGTGCTAG TCAG   34

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 132 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

```
GCTAGAATAT CAAGCCAGAA AGTGCTCGCT GACTGGGAAA          40
TGGACCAACG ATCTGGGCTC CAACATGACC ATCGGGGCTG          80
TGAACAGCAG AGGTGAATTC ACCCTGCGCA CACAGAAGGA         120
GCGGTGCAAC AC                                        132
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Claims

We claim:

1.   A method of killing tumor cells *in vivo*, comprising transducing cells in or adjacent to a tumor with a gene delivery vehicle containing a nucleic acid molecule encoding for a polypeptide capable of stimulating blood clot formation in or adjacent to the tumor.

2.   A method of inhibiting tumor angiogenesis *in vivo*, comprising transducing cells in or adjacent to the tumor with a gene delivery vehicle that comprises a nucleic acid molecule encoding for a polypeptide capable of inhibiting vascularization of the tumor.

3.   A method of killing tumor cells *in vivo*, comprising:

       (a)   transducing cells of a blood vessel in or adjacent to an arterial side of a tumor with a gene delivery vehicle that comprises a nucleic acid molecule encoding a polypeptide capable of activating a non-cytotoxic agent to a cytotoxic agent; and

       (b)   administering to the animal a non-cytotoxic agent capable of being activated by the polypeptide into a cytotoxic agent.

4.   A method of depriving tumor cells *in vivo* of nutrients, comprising transducing cells in or adjacent to a blood vessel in a tumor with a gene delivery vehicle comprising a nucleic acid molecule encoding a polypeptide capable of binding, metabolizing, or rendering resistant to cellular uptake, nutrients in the perivascular interstitial space of the tumor.

5.   A method according to any one of claims 1, 2, 3, or 4 wherein the gene delivery vehicle is a recombinant retroviral vector.

6.   A method according to claim 1 wherein the gene delivery vehicle containing a nucleic acid molecule encoding a polypeptide capable of stimulating blood clot formation.

7.   A method according to claim 6 wherein the polypeptide is selected from the group consisting of Russell's viper venom factor X-activating factor, Russell's viper venom factor V-activating factor, tissue factor, truncated tissue factor, a truncated tissue factor-vascular endothelial growth factor fusion protein, cancer procoagulant, thrombin, and a thrombin-like enzyme.

8.   A method according to claim 6 wherein the polypeptide is selected from the group consisting of von Willebrand factor antigen II, endothelial-monocyte-activating polypeptide I, endothelial-monocyte-activating polypeptide II, tumor necrosis factor α, tumor necrosis factor β, and the tissue factor-inducing factor from *Rickettsia* rickettsii.

9.   A method according to claim 8 wherein the thrombin-like enzyme is selected from the group consisting of thrombin-like enzymes from the venoms of *Crotalus* adamanteus (Crotalase), *Crotalus* horridus horridus, *Agkistrodon* Rhodostoma (Ancrod), *Agkistrodon* contortrix contortrix, *Agkistrodon* acutus, *Bothrops* atrox (Batroxobin), *Bothrops* marajoensis, *Bothrops* moojeni, *Trimeresurus* gramineus, *Trimeresurus* okinavensis and *Bitis* gabonica.

10.   A method according to claim 1 wherein the gene delivery vehicle comprises a nucleic acid molecule encoding a polypeptide capable of inhibiting fibrinolysis.

11.   A method according to claim 7 wherein the polypeptide is selected from the group consisting of α2-antiplasmin, plasminogen activator inhibitor I, plasminogen activator inhibitor II, plasminogen activator inhibitor III and *Erythrina* proteinase inhibitors.

12.   A method according to claim 2 wherein the recombinant retroviral vector comprises a nucleic acid molecule encoding a polypeptide capable of inhibiting tumor vascularization.

13.   A method according to claim 12 wherein the polypeptide is selected from the group consisting of angiostatin, interferon α, interferon β, platelet factor-4, tissue inhibitors of metalloproteinases I, tissue inhibitors of metalloproteinases II, tissue inhibitors of metalloproteinases III, thrombospondin, the anti-angiogenic fragment of prolactin, heparinase, neutralizing antibody fragments against basic fibroblast growth factor, vascular endothelial cell growth factor and αVβ3 integrin.

14.   A method according to claim 3 wherein the gene delivery vehicle comprises a nucleic acid molecule encoding a polypeptide capable of activating a non-cytotoxic agent into a cytotoxic agent.

15.   A method according to claim 3 wherein the polypeptide is selected from the group consisting of *Herpes* simplex virus thymidine kinase, varicella-zoster virus thymidine kinase, cytosine deaminase, *Escherischia* coli purine nucleoside phosphorylase, Leishmania purine nucleoside phosphorylase, *Escherischia* coli xanthine-guanine phosphoribosyl transferase, the cytochrome P450 2B1 gene product, cytochrom p450 reductase cell surface, alkaline phosphatase, β-glucosidase, N-deoxyribosyl transferase, ferrodoxin oxidoreductase, carboxypeptidase G2, carboxypeptidase A, β pactamase, actinomycin D synthetase complex, and nitroreductase.

16.   A method according to claim 4 wherein the gene delivery vehicle comprises a nucleic acid molecule encoding a polypeptide capable of binding, metabolizing, or rendering resistant to cellular uptake, nutrients in the perivascular interstitial space of the tumor.

17.   A gene delivery vehicle containing a nucleic acid molecule encoding a polypeptide capable of stimulating clot formation.

18.   A gene delivery vehicle containing a nucleic acid molecule encoding a polypeptide capable of inhibiting fibrinolysis.

19.   A gene delivery vehicle containing a nucleic acid molecule coding for a polypeptide capable of inhibiting tumor vascularization.

20.   A gene delivery vehicle containing a nucleic acid molecule encoding a polypeptide capable of binding, metabolizing, or rendering resistant to cellular uptake, nutrient in the perivascular interstitial space of the tumor.

21.   A producer cell that produces a gene delivery vehicle according to claim 17.

22.   A producer cell that produces a gene delivery vehicle according to claim 18.

23.   A producer cell that produces a gene delivery vehicle according to claim 19.

24.   A producer cell that produces a gene delivery vehicle according to claim 20.

25.   A target cell transduced with a gene delivery vehicle of claim 17.

26.   A target cell transduced with a gene delivery vehicle of claim 18.

27.   A target cell transduced with a gene delivery vehicle of claim 19.

28.   A target cell transduced with a gene delivery vehicle of claim 20.

29.   A target cell transduced with a gene delivery vehicle of any of claims 17, 18, 19, or 20 wherein the recombinant vector is derived from a virus selected from the group consisting of an adenovirus, a retrovirus, a pox virus, an alphavirus, a poliovirus, a rhinovirus, an influenza virus, a parvovirus, an adeno-associated virus, a *Herpes* virus, a SV40 virus, a human immunodeficiency virus, a measles virus, an astrovirus, and a corona virus.

30.   A gene delivery vehicle of claims 1, 2, 3, or 4 wherein the gene delivery vehicle is selected from the group consisting of a viral vector, a nucleic acid vector, a liposome, a polycation condensed nucleic acid or a recombinant vector.

31.   A gene delivery vehicle produced by the producer cell of claim 21.

32.   A gene delivery vehicle produced by the producer cell of claim 22.

33.   A gene delivery vehicle produced by the producer cell of claim 23.

34.   A gene delivery vehicle produced by the producer cell of claim 24.

35.   A gene delivery vehicle according to any one of claims 31, 32, 33, or 34 wherein the gene delivery vehicle is selected from the group consisting of an adenoviral particle, a retroviral particle, a poxviral particle, an alphaviral particle, a polioviral particle, a rhinoviral particle, an influenza viral particle, a parvoviral particle, an adeno-associated viral particle, a herpes viral particle, a SV40 viral particle, a human immunodeficiency viral particle, a measles viral particle, an astroviral particle, and a corona viral particle.

36.   A gene delivery vehicle according to claim 35 wherein the alphaviral particle is selected from the group consisting of Sindbis virus, Semliki Forest virus, Middleberg virus, Ross River virus, and Venezuelan equine encephalitis virus.

37.   A retroviral particle according to claim 35 wherein the gene delivery vehicle is selected from the group consisting of avian leukosis virus, bovine leukemia virus, murine leukemia virus, mink-cell focus-inducing virus, murine sarcoma virus, reticuloendotheliosis virus, gibbon ape leukemia virus, Mason-Pfizer leukemia virus and rous sarcoma virus.

38.   A murine retroviral particle according to claim 37 wherein the retroviral particle is selected from the group consisting of Abelson, Friend, Graffi, Gross, Kristen, Harvey sarcoma, raucher and Moloney murine leukemia.

39.   A gene delivery vehicle according to any one of claims 31, 32, 33, or 34 wherein the viral particle is replication defective.

40.   A pharmaceutical composition comprising a gene delivery vehicle of any of claims 31, 32, 33, or 34.

41.   A gene delivery vehicle of any one of claims 31, 32, 33, or 34 that is lyophilized.

42.   A gene delivery vehicle according to claim 35 that is lyophilized.

43.   A lyophilized gene delivery vehicle according to claim 42 wherein the gene delivery vehicle upon reconstitution is suitable for administration to humans.

44.   A gene delivery vehicle of any one of claims 31, 32, 33, or 34 that is dehydrated.

45.   A gene delivery vehicle according to claim 35 that is dehydrated.

FACTOR	NAME
I	FIBRINOGEN
II	PROTHROMBIN
III	TISSUE FACTOR
IV	CALCIUM
V	PROACCELERIN, LABILE FACTOR
VII	PROCONVERTIN, STABLE FACTOR
VIII	ANTIHEMOPHILIC A FACTOR (AHF), ANTIHEMOLPHILIC GLOBULIN (AHG)
IX	ANTIHEMOPHILIC B FACTOR (AHB), PLASMA THROMBOPLASTIN COMPONENT (PTC), CHRISTMAS FACTOR
X	STUART FACTOR, STUART-POWER FACTOR
XI	PLASMA THROMOBOPLASTIN ANTECEDENT(PTA)
XII	HAGEMAN FACTOR, CONTACT FACTOR
XIII	FIBRIN STABILIZING FACTOR
–	FLETCHER FACTOR, PREKALLIKREIN
–	HIGH MOLECULAR WEIGHT KININOGEN, HMWK, FITZGERALD FACTOR
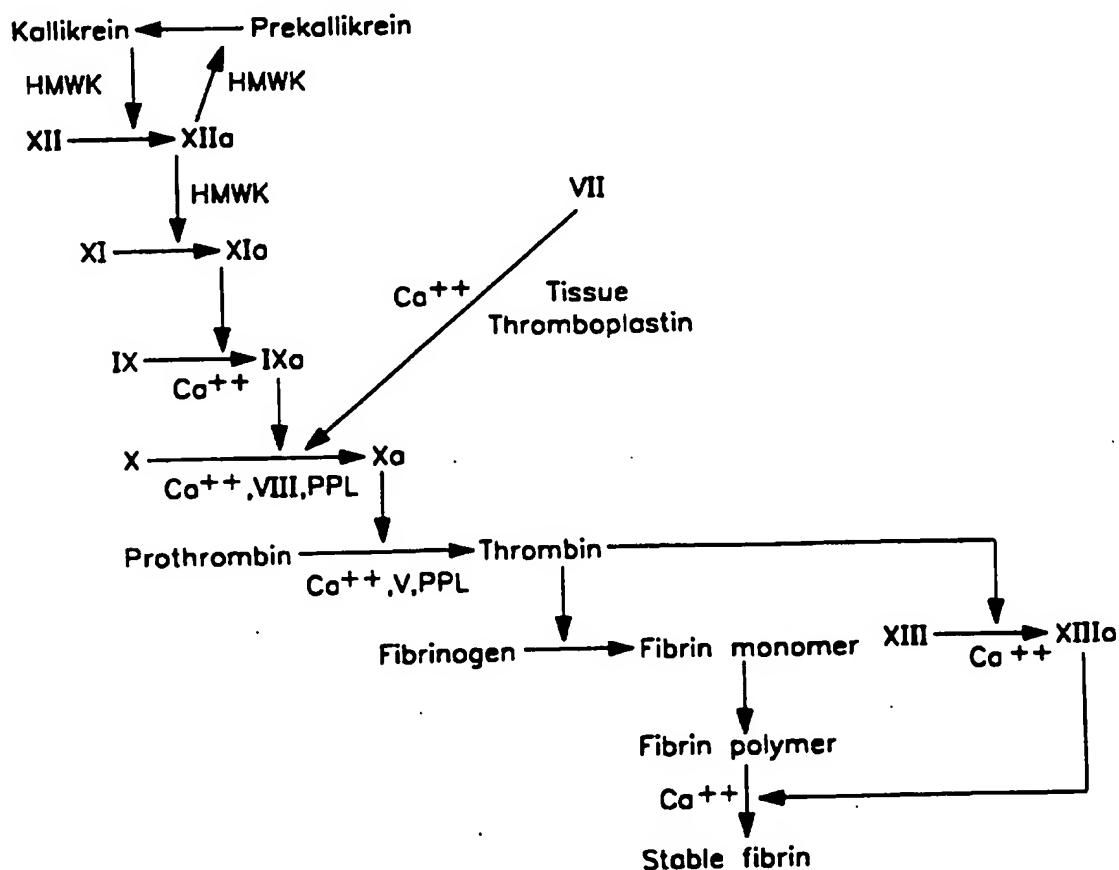
PPL=Platelet phospholipid

FIG. I

FIG. 2

FIG. 3

FIG. 5

FIG. 6

FIG. 7

Bgl II   Ava I   Ava I   Ava I BamHI

0.3 Kb   0.64 Kb   0.22 Kb

Bgl II   BamHI

A   R   E   M   G   E   A   N   ...
CCC GA/GAG/ATG/GGG/GAG/GCT/AAC/TGAG
GGG CT CTC TAC CCC CTC CGA TTG ACACCTAG

Altered from C / G in wild type ⟹ lose SmaI site, keep AvaI site

5' end of SmaI site

Xho Bgl II BssH II   Nco I ≡ AvaI

400   420   440   480

Hinc II
Acc I

Eco RI   Kpa I   Bam HI   Sal I   Sph I

agtgaattCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGcgtactcatggtcat

SacI   SmaI   Xhol   PstI   HindIII
      Xmal

Lac Z ← Thr Ile Met Thr(Met)

Ssp I 2501   Nde I 183   Polylinker cloning site 396-447

LacZ

ScaI   2177

Ap

Ampr

TKΔA
3.9 Kb

Lac

ORI

FIG. 8

FIG. 9

FIG. IO

FIG. I IA

FIG. I IB

PROMOTER	5' ALPHAVIRUS NONSTRUCTURAL PROTS.	JR	G.O.I	3' SEQ	TERM/pA

RNA(+) ——————————————————————————————————►

NUCLEUS
_____
　　　　　　　　　　TRANSPORT
_____
CYTOPLASM

RNA(+) ——————————————————————————————————►

ALPHAVIRUS POLYMERASE PROTEINS

RNA(−) ◄———————————————————————

AUTOCATALYTIC EVENT

RNA(+) ——————————————————————————————————►

RNA(+) ——————————————►
——————————————►
——————————————►
——————————————►
——————————————►

GOI PROTEIN

FIG. 12

.1 2 / 2 8

FIG. 13

FIG. 14

FIG. 15

FIG. 16

FIG. 17

FIG. 18

FIG. 19

Formulation:
　　　　25mM Tris pH 7.2
　　　　60mM NaCl
　　　　1 mg/ml Arginine
　　　　5 mg/ml HSA
　　　　50 mg/ml Lactose

Legend:
- ---•--- -80°C Liquid
- —○— -20°C Liquid
- —▽— -20°C Lyoph
- ---■--- Refrig. Lyoph
- ---▲— R.T. Lyoph

FIG. 20
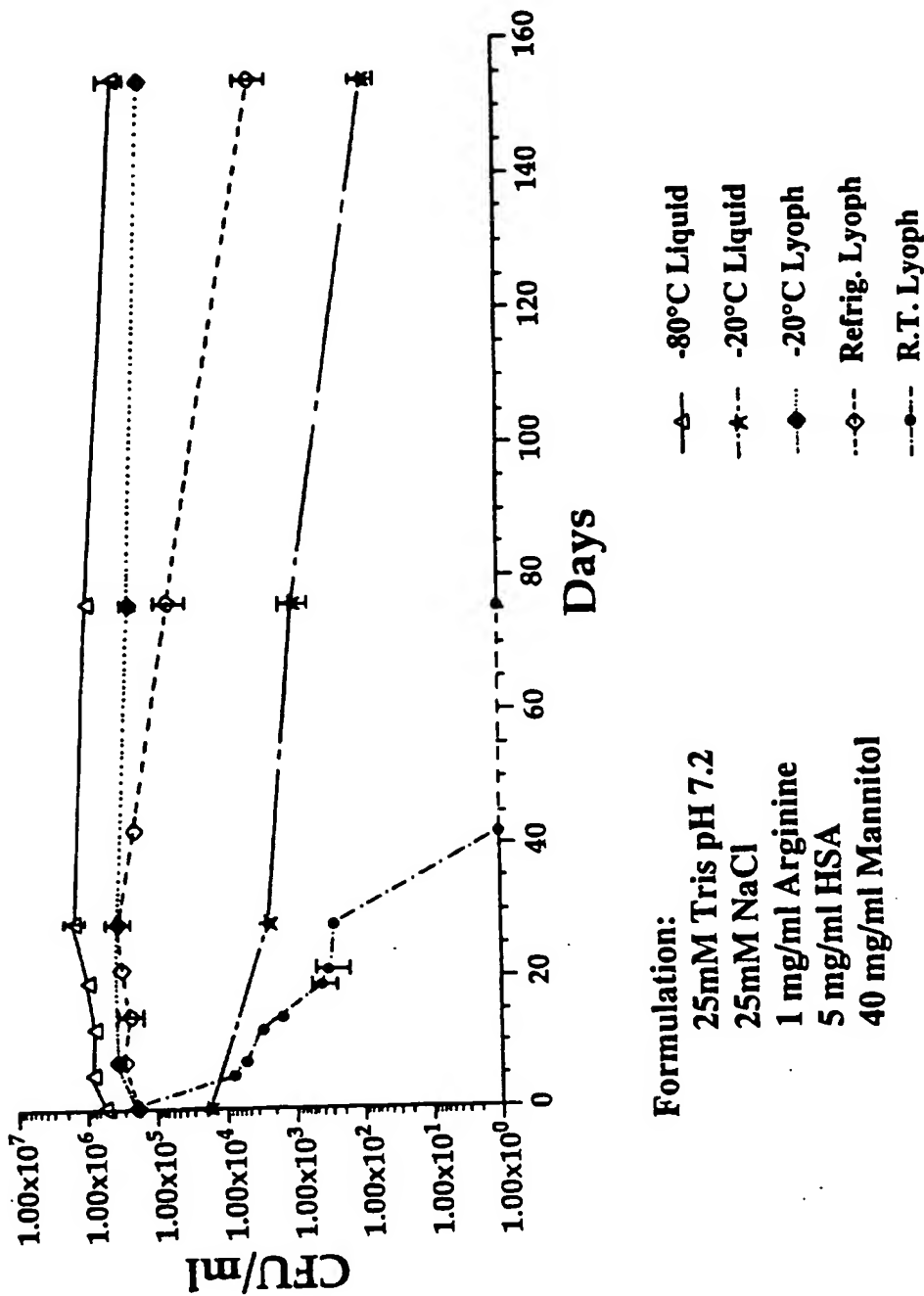
FIG. 21
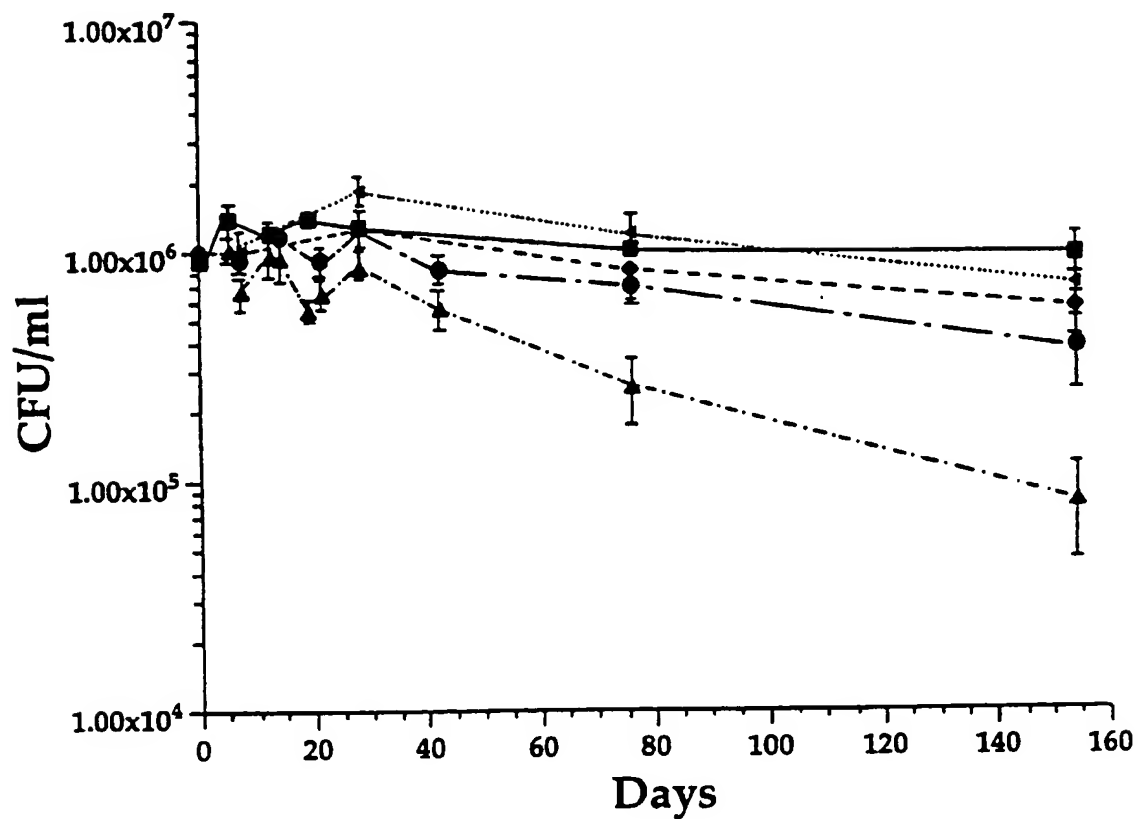
Formulation:
    25mM Tris pH 7.2
    60mM NaCl
    1mg/ml Arginine
    5mg/ml HSA
    50mg/ml Trehalose

    —■— -80°C Liquid
    ····◄·· -20°C Liquid
    --◆·· -20°C Lyoph
    —●— Refrig. Lyoph
    —▲— R.T. Lyoph

FIG. 22
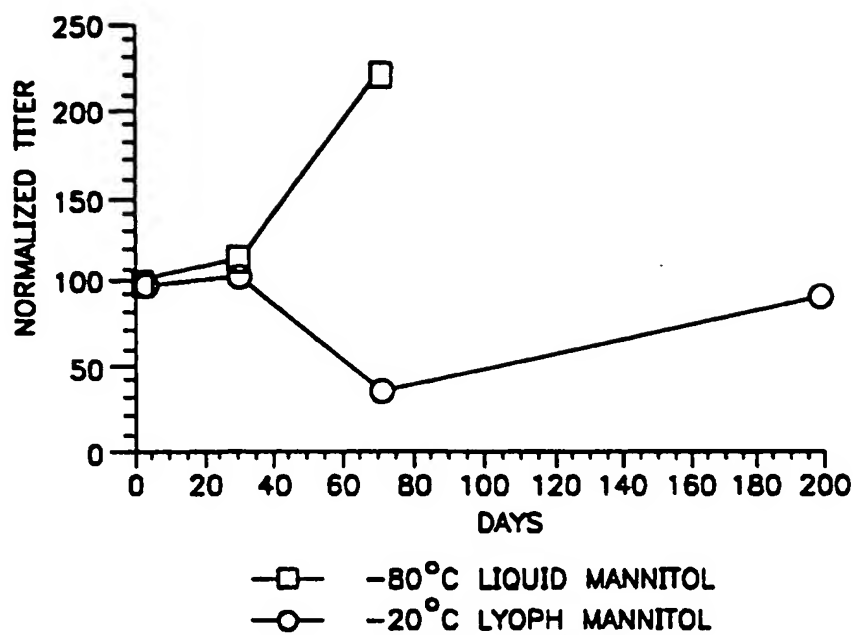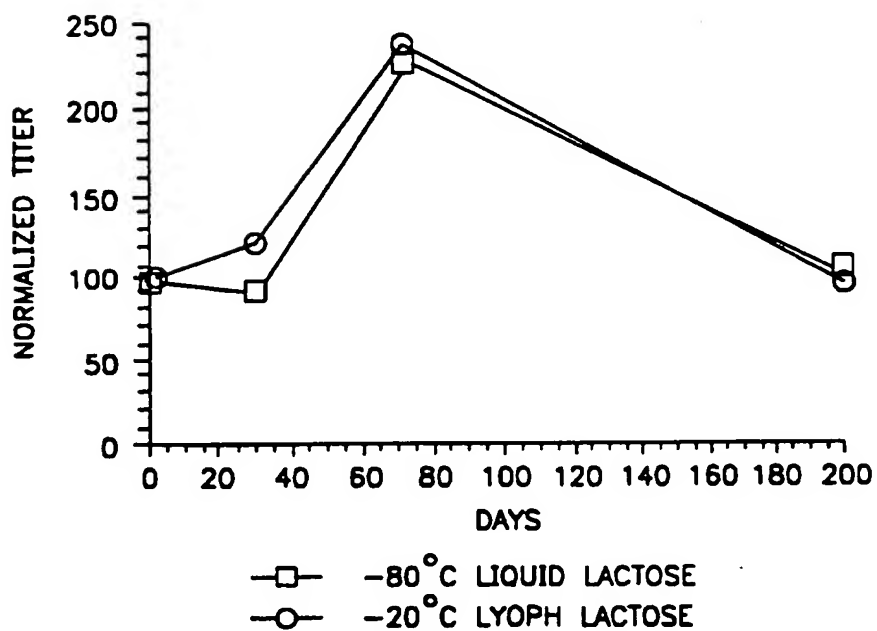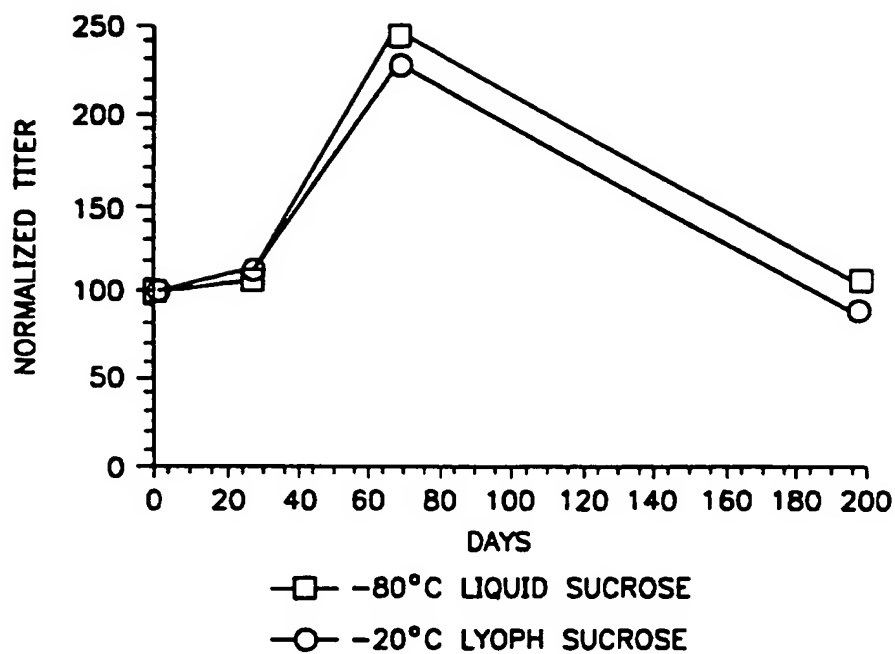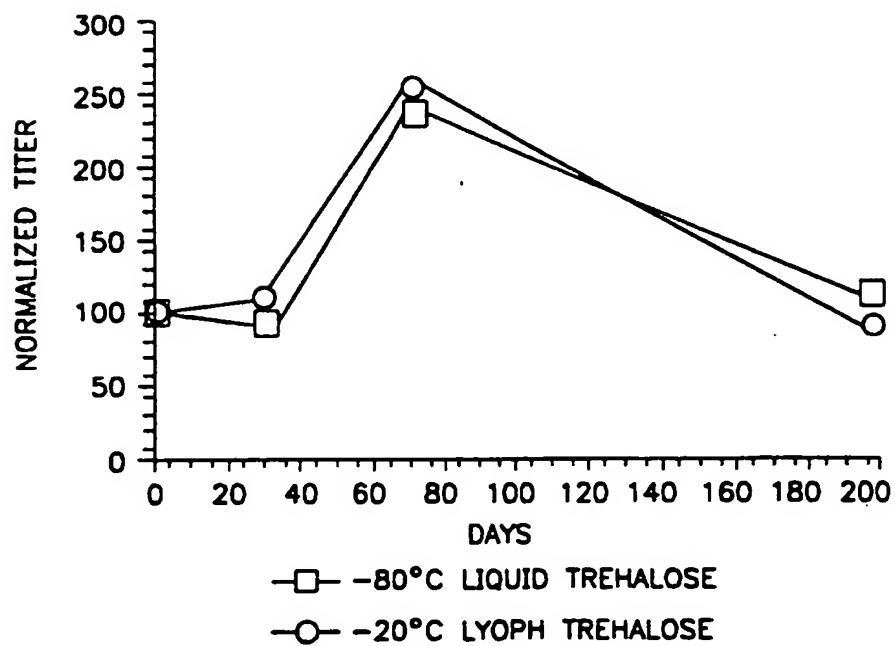
FIG. 23A

FIG. 23B

FIG. 23C

FIG. 23D
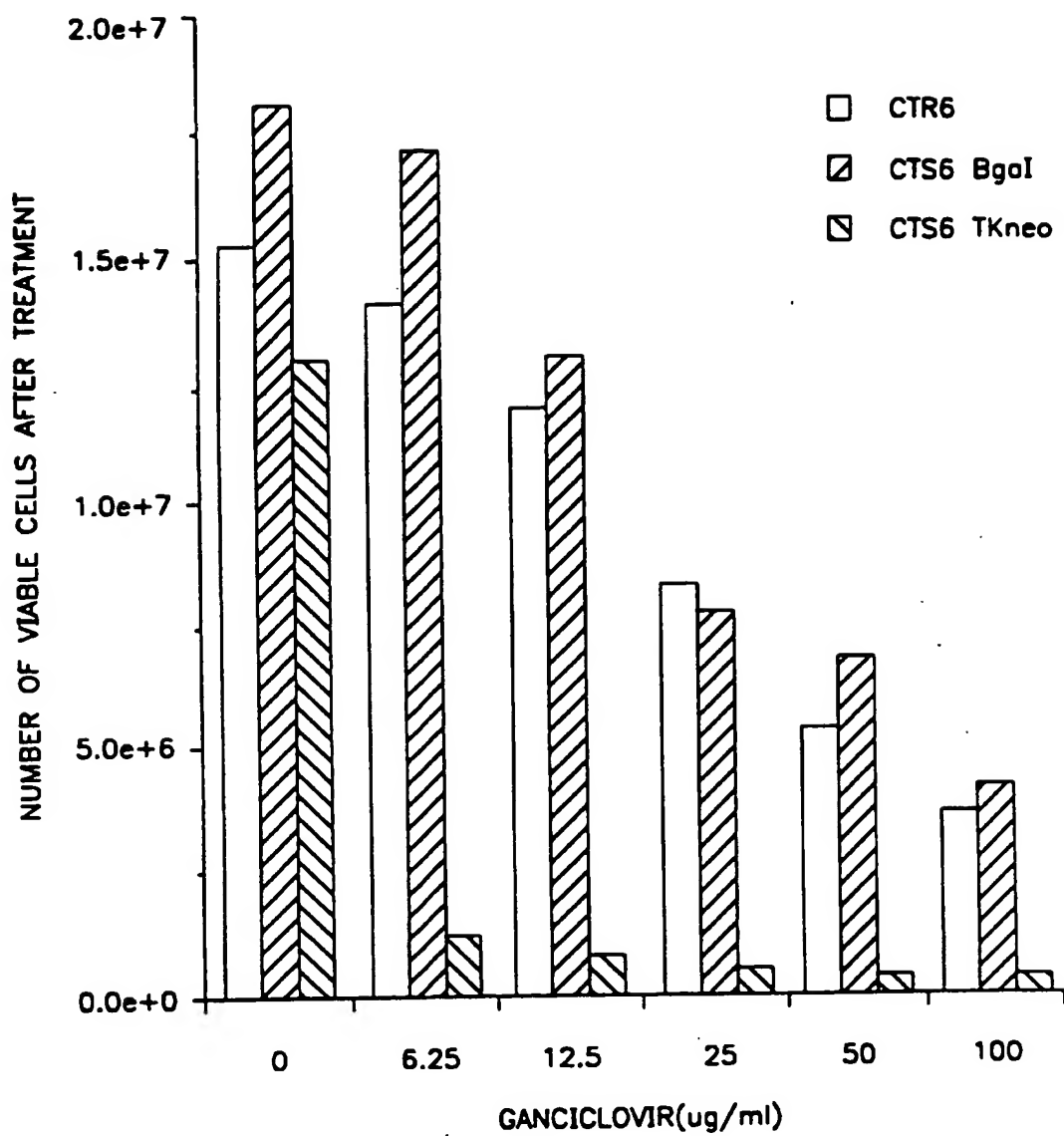
FIG. 24
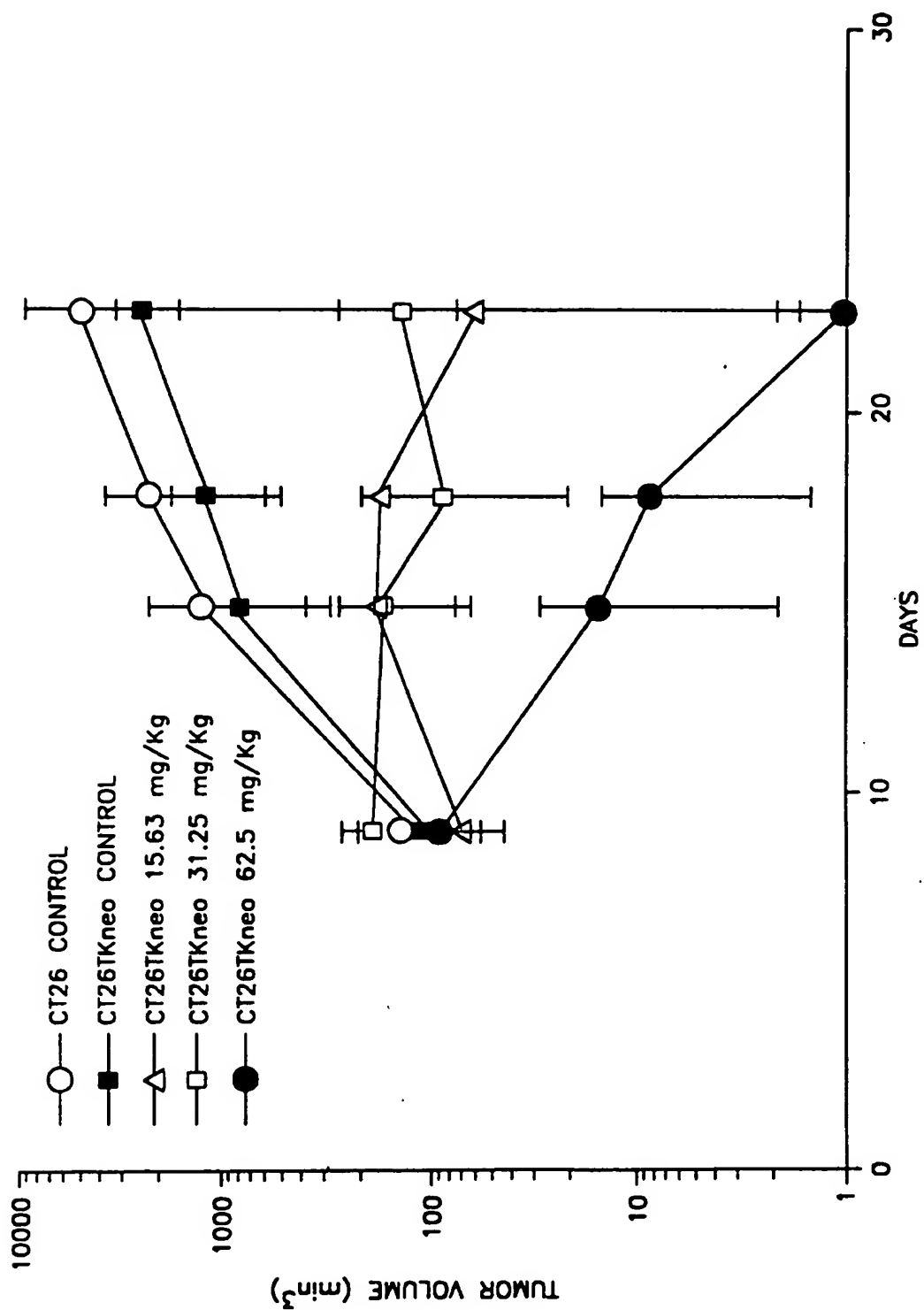
FIG. 25
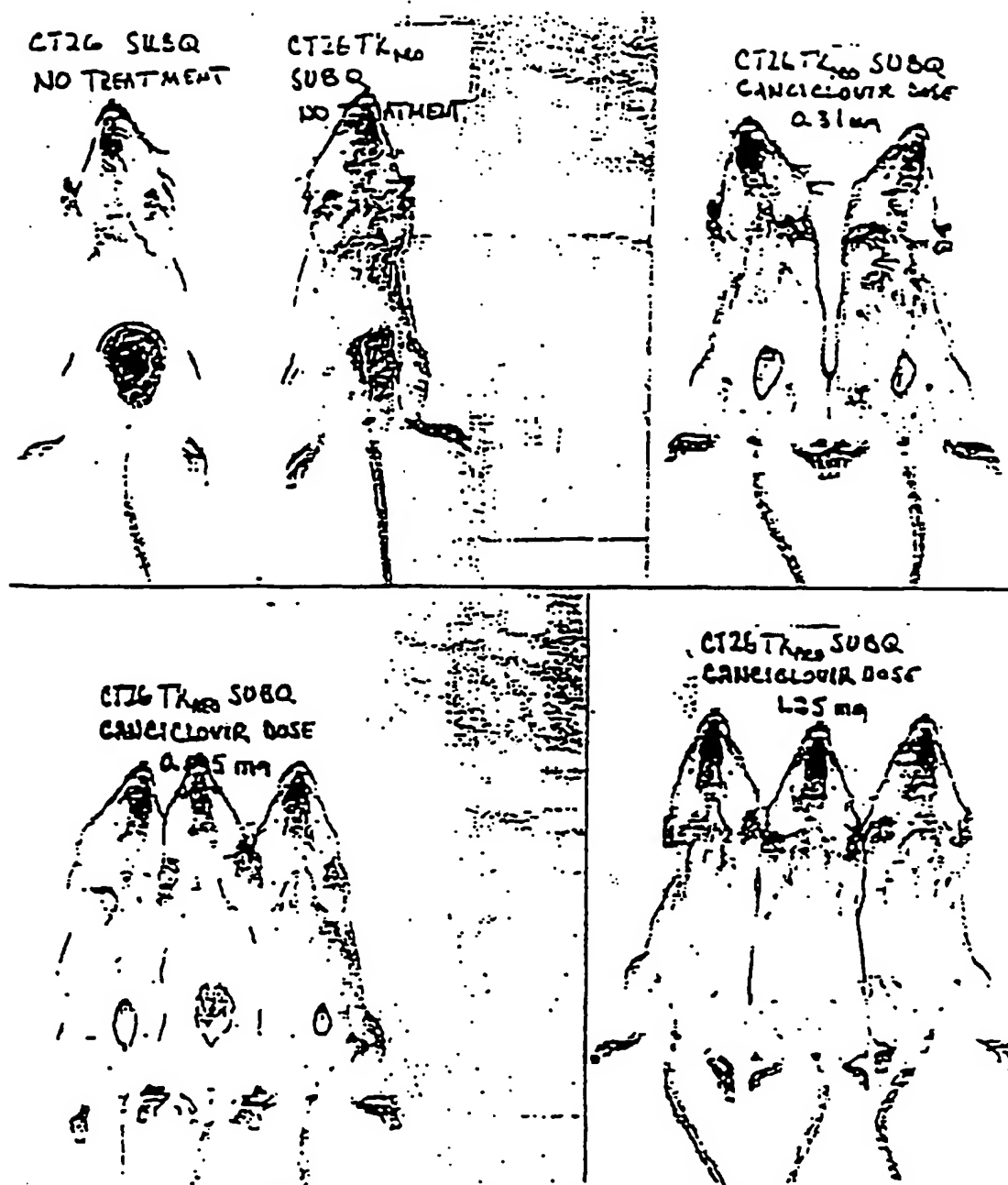
Figure 26

The Effect of Different Dose Regimen of Ganciclovir on Intraperitoneal
CT26TKNeo Tumor Growth

Figure 27
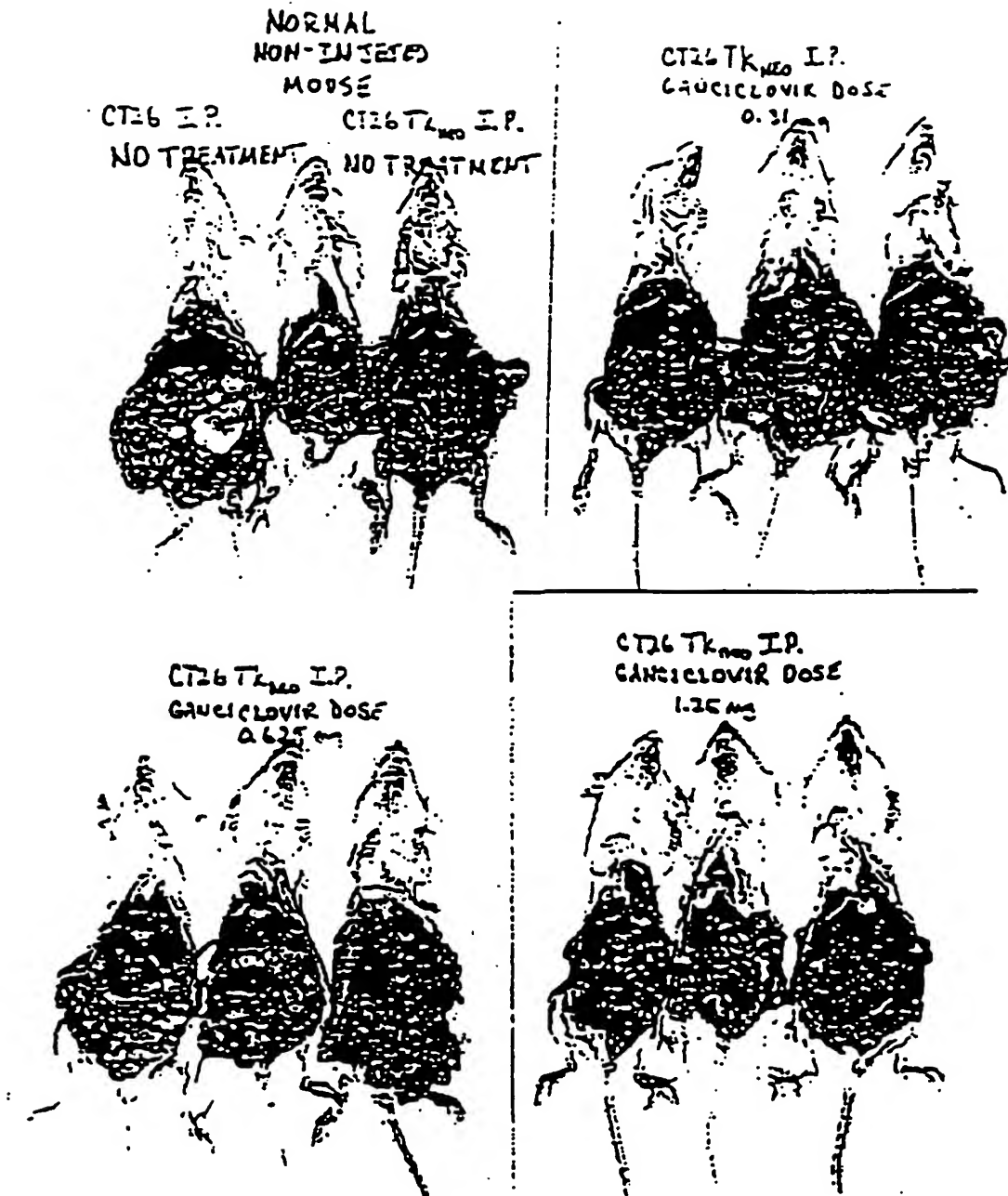
The Effect of Different Dose Regimen of Ganciclovir on Subcutaneous
CT26TKNeo Tumor Growth

FIG. 28